Effects of Sample Solvent Composition and Injection Volume on Chromatographic Peak Profiles of Methyl 2-Benzimidazolecarbamate and 3-Butyl-2,4-dioxo[1,2-a]-s-triazinobenzimidazole in RP-HPLC

Donna Vukmanic, B. Sc. (Honours)

A thesis submitted to the
Department of Chemistry
in partial fulfilment of the requirements
for the degree of
Master of Science

Brock University,
St. Catharines, Ontario
November, 1987

To my husband Nick and son Mark

for their understanding and encouragement

ABSTRACT

The effects of sample solvent composition and the injection volume, on the chromatographic peak profiles of two carbamate derivatives, methyl 2-benzimidazolecarbamate (MBC) and 3-butyl-2,4-dioxo[1,2-a]-s-triazinobenzimidazole (STB), were studied using reverse phase high performance liquid chromatograph. The study examined the effects of acetonitrile percentage in the sample solvent from 5 to 50%, effects of methanol percentage from 5 to 50%, effects of pH increase from 4.42 to 9.10, and effect of increasing buffer concentration from 0 to 0.12M. The effects were studied at constant and increasing injection mass and at four injection volumes of 10, 50, 100 and 200 µL. The study demonstrated that the amount and the type of the organic solvents, the pH, and the buffer strength of the sample solution can have a pronounced effect on the peak heights, peak widths, and retention times of compounds analysed. MBC, which is capable of intramolecular hydrogen bonding and has no tendency to ionize, showed a predictable increase in band broadening and a decrease in retention times at higher eluting strengths of the sample solvent. STB, which has a tendency to ionize or to strongly interact with the sample solvent, was influenced in various ways by the changes in ths sample solvent composition. The sample solvent effects became more pronounced as the

injection volume increased and as the percentage of organic solvent in the sample solution became greater. The peak height increases for STB at increasing buffer concentrations became much more pronounced at higher analyte concentrations. It was shown that the widely accepted procedure of dissolving samples in the mobile phase does not yield the most efficient chromatograms. For that reason samples should be dissolved in the solutions with higher aqueous content than that of the mobile phase whenever possible. The results strongly recommend that all the samples and standards, regardless whether the standards are external or internal, be analysed at a constant sample composition and a constant injection volume.

Acknowledgements

The author would like to express her sincere gratitude and appreciation to Dr. M. Chiba for his guidance and patience during the course of this work.

Thanks are also extended to Dr. H. L. Holland, Dr. J. M. Miller and Tim Jones for their advice and helpfull suggestions.

Thanks are due to D. Legare, B. McGarvey and R. Richards for technical assistance.

I would also like to thank Mrs. Olga Bukovec and the rest of the chemistry department for their support and encouragement.

Table of Contents

List of Tables

List of Figures

I. Introduction

In the last decade High Performance Liquid Chromatography (HPLC) has become one of the most widely used analytical techniques for a variety of purposes. This is due in part to the introduction of sophisticated instrumentation but is primarily due to the methodology available to separate, identify and quantitate complex mixtures of compounds in a relatively short period of time.

A. History of Chromatography

In 1906 botanist Michael S. Tswett published the first paper (1) on chromatographic separation and coined the name "Chromatography". He described how components of a mixture (plant pigments) can be separated by elution through a packed column because of their different affinities for the column adsorbent. His work, however, was not widely recognized and chromatography held no significant importance until 1941 when Martin and Synge (2) published their historical paper on chromatography which led to the 1952 Nobel prize in Chemistry. The four major aspects of their paper were: 1) Theoretical treatment of separation process by expressing the efficiency of the column in terms of theoretical plate height; 2) Description of partition chromatography; 3) Recognition that a liquid moving phase can be replaced by a gas; 4) Recognition that the most efficient columns can be obtained by using very

small particles and by applying high pressure differences across the column.

Chromatography has developed in stages since that time. Partition and paper chromatography were important developments in the 1940s, gas and thin-layer chromatography in the 1950s and gel or exclusion chromatography in the early 1960s. Modern High Performance Liquid Chromatography (HPLC) is only 18 years old. The first commercial equipment and column packings could be purchased in 1969. This was much later than for gas chromatography. The fast speed of development and improvements in this technique since then is due to several factors: 1) Thorough understanding of Gas Chromatographic (GC) theory; 2) Operational advantages over traditional column chromatography, thin layer and paper chromatography in terms of speed, accuracy, convenience and the ability to separate complex mixtures (3-5). 3) Wide range of applicability. While gas chromatography is limited to separating only 20 percent of known organic compounds without further derivatization (6) (since the rest are either insufficiently volatile or thermally unstable), HPLC can separate any compound that is soluble in a liquid suitable for use as a mobile phase. It has found a wide range of applications in the biomedical (7), natural product (8), enviromental (9,10) and polymer (11) fields. It is used in analysis and separation of proteins, nucleic acids, amino acids, polysaccharides, plant pigments, lipids, pharmaceutical products, drugs, pesticides, steroids, vitamins, flavor enhancers, enviromental pollutants and

polymers.

B. HPLC Instrumentation

The basic principles of HPLC and classical column chromatography are similar. In both, physical separation of different molecular species occurs on the packed column using liquid as a mobile phase. HPLC, however, employs much more sophisticated equipment, which allows separation at high pressure (usually up to 40 megapascals) and constant and reproducible flow (usually from 0.01 to 10 mL/min) using long life, reusable columns. These factors are responsible for major HPLC advantages of convenience, accuracy, speed of analysis and the ability to carry out complex separations. The basic HPLC set-up is illustrated in Figure 1. The main components are:

(1) Degassed solvent reservoir. Degassing is usually required to avoid pressure fluctuations.

(2) Pump system. It is capable of delivering pulseless and reproducible mobile phase flow usually in the range from 0.01 to 10 mL/min. It must be compatible with organic and aqueous solvent systems. The two piston reciprocating pumps with pulse dampeners are most popular at present. Other types include syringe-type pumps, pneumatic pumps and hydraulic amplifier pumps.

(3) High-pressure injector. There are four basic types: high-pressure septum injector,high-pressure stop-flow

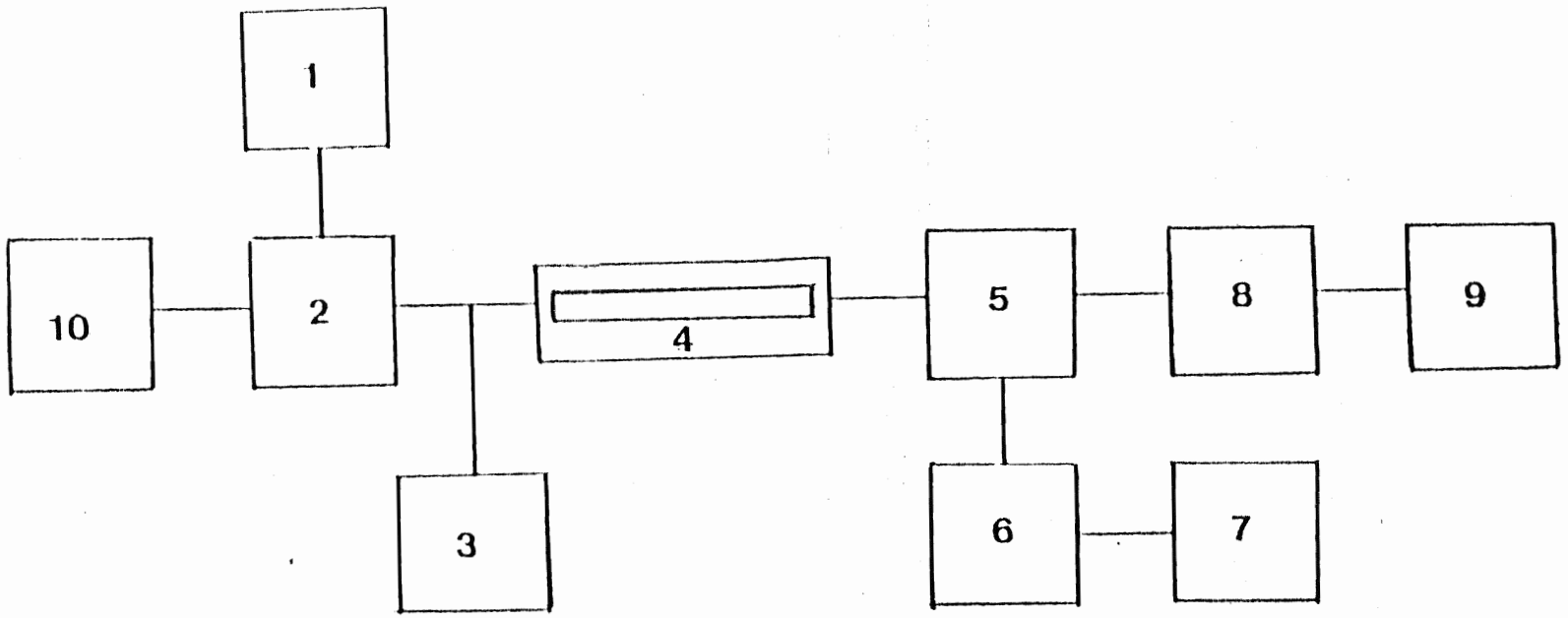


Figure 1: Block Diagram of Liquid Chromatograph: 1) Mobile Phase Reservoir; 2) Pump; 3) High Pressure Injector; 4) Thermostated Stainless Steel Column; 5) Detector; 6) Detector Electronics; 7) Recorder; 8) Sample Collection; 9) Flow Measurement; 10) Programmer.

injector, loop injector and syringe-loop injector. The syringe-loop type seems to be the most widely used.

(4) Column. The most commonly used column is a closed, usually stainless steel, column from 5 to 30 cm in length and 1 to 4.6 mm in internal diameter, capable of withstanding pressures up to 40 megapascals. It is packed with uniform small size particles (3-10 μm) and is connected with narrow bore tubing to the injector and detector to minimize dead volume which causes band broadening.

(5) Detector. It is used to identify and quantitate separated components. Three major requirements are high sensitivity (10^{-6}-10^{-9} g/mL range), high signal to noise ratio, and large linear response range. Most widely used are refractive index, UV absorption, fluorometric and conductivity detectors. With the introduction of microbore columns (12), infra red (13), electrochemical (14) and mass spectrometric detectors (15,) are gaining in importance.

C. Theory of Chromatographic Separation

Chromatographic separations can be obtained when there is a difference in specific affinities of solute components for the stationary phase and moving phase. Figure 2 illustrates several steps in the separation process. In step 1, the sample is introduced through an injection port into the continuously flowing mobile phase stream. A tight band of solute molecules is formed at the head of the column. In step

Figure 2: Steps in Chromatographic Separation: 1) Sample Intorduction; 2) Chromatographic Separation; 3) Sample Detection.

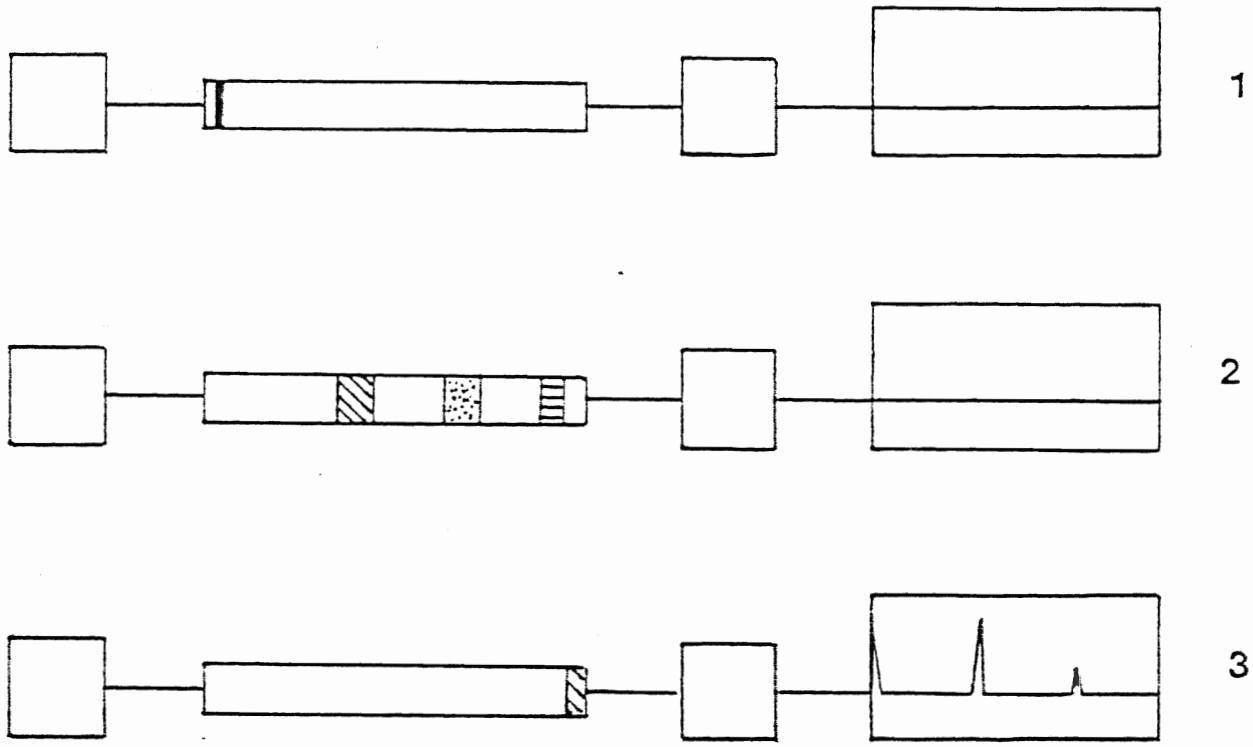
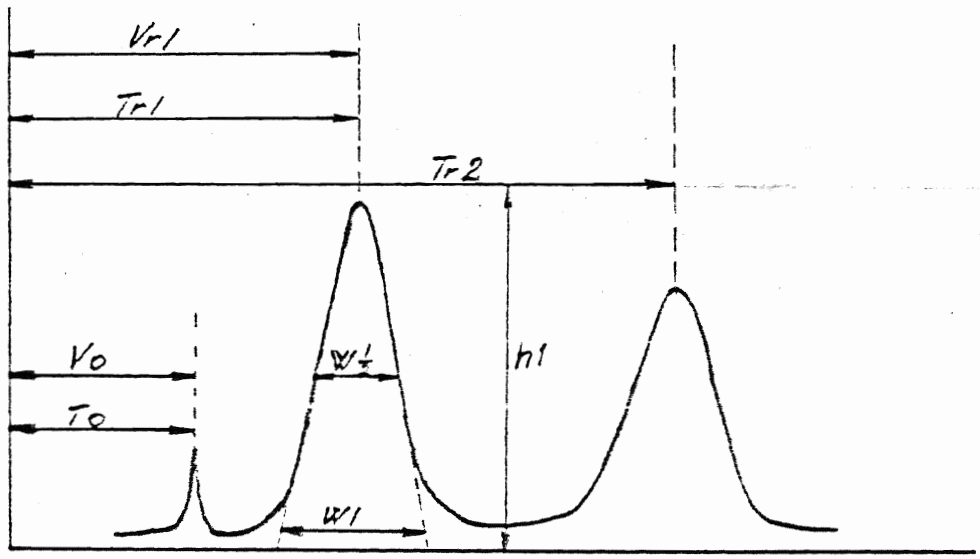


Figure 3: Major Parameters of a Chromatogram: T_o = dead time, V_o = dead volume, T_{r1} = retention time for component 1, V_{r1} = retention volume for component 1, T_{r2} = retention time for component 2, h_1 = peak height for component 1, w_1 = peak width for component 1, $w_{1/2}$ = peak width at half height.



2, sample is carried down the column and due to the different interaction of each component with the stationary and moving phases, each component travels at a different speed down the column. There is also some band broadening present, due to undesirable diffusion processes occurring in the column during the separation. Since the injection solvent is not retained by the stationary phase, it travels down the column at the same rate as the mobile phase flow and always elutes first at retention time To. In step 3, the separated components elute from the column and enter the detector, which sends a response to a constant speed strip-chart recorder, or to a computer terminal. Results are plotted in the form of detector response versus the time taken for each component to be eluted (retention time) Tr(i). This type of plot is known as a chromatogram.

Figure 3 illustrates a typical chromatogram with the parameters used to characterize it. Each component elutes independently of others from the column and can be described by the set of independent variables: dead volume Vo or dead time To, retention volume Vr(i) or retention time Tr(i), peak width W(i), and peak height h. From the above parameters the capacity factor k'(i) can be calculated using equation [1]:

$$k'(i) = [Vr(i)-Vo]/Vo \qquad \text{eq. [1]}$$

or if the eluent flow rate is constant, the retention times can be used in place of Vr as shown in equation [2]:

$$k'(i) = [Tr(i)-To]/To \qquad \text{eq. [2]}$$

Capacity factor k'(i) is one of the most important factors in chromatography. It relates equilibrium distribution of the sample to the thermodynamic properties of the column and can also be described by equation [3]:

$$k'(i) = Cs(i)Vs/Cm(i)Vm \qquad \text{eq. [3]}$$

where Cs(i) = concentration of the solute in the stationary phase, Cm(i) = concentration of the solute in the mobile phase, Vs = volume of the stationary phase in the column, Vm = volume of the mobile phase in the column. The ratio Cs(i)/Cm(i) = Kd, is known as a distribution coefficient. Kd is constant for a specific system if the temperature is kept constant and the distribution isotherm is linear, giving symmetrical elution peaks. When the distribution isotherm deviates from linearity, elution profiles become asymmetric with peak tailing or leading edges appearing (16). Peak tailing indicates adsorptive effects and retention time for the components decreases with increasing sample size. A leading edge indicates a concave isotherm. Here, increasing sample size increases component retention time, and symmetrical peaks can be achieved by decreasing the sample load (16). Since the above values are difficult to obtain during chromatographic analysis, equations [1] and [2] are used most of the time to calculate k'. Another important separation factor is resolution Rs between the two adjacent

bands. It is determined by the performance of chromatographic system which is influenced by the choice of mobile and stationary phases, temperature, and length of the column. Resolution of 1.5 or higher indicates complete separation. It can be readily calculated from equation [4]:

$$Rs = [T(r1)-T(r2)]/[1/2(W_2+W_1)] \qquad \text{eq. [4]}$$

Chromatographic separation can also be considered as a series of extractions, as mobile phase moves past the stationary phase. The single equilibrium extraction occurs over a specific length of the column and is known as a theoretical plate. The number of theoretical plates (N), which exhibits the efficiency of the column, characterizes the entire chromatographic separation system, and is a convenient measure to assess the band spreading. N is determined from equation [5]:

$$N = 5.52\,[Tr(i)/W(i)\text{at } h_{1/2}]^2 = 16[Tr(i)/W(i)]^2 \qquad \text{eq. [5]}$$

Efficiency can also be expressed as the height equivalent to a theoretical plate (HETP) from equation [6]:

$$H = L/N \qquad \text{eq. [6]}$$

Smaller H means better column efficiency and smaller band spreading. By comparing the theoretical plate heights of two columns, one can get direct measure of relative column

efficiency. The factors that affect the degree of band spreading are given by the Van Deemter equation [7] (17) :

$$HETP=1/[(1/C_e d_p)+(1/C_m d_p^2 u/D_m)]+C_d D_m/u+C_{sm} d_p^2 u/D_m \quad \text{eq. [7]}$$

where d_p is the diameter of column packing particles, u is the mobile phase velocity inside the column, D_m and D_s are molecular diffusion coefficients of the sample in the moving and stationary phases respectively, and C_e, C_m, C_{sm}, C_s, C_d are constants characteristic of the packing. Equation [7] states that smaller HETP and improved separations are favoured as particle size and mobile phase velocity decrease, as mobile phase solvents become less viscous, as separation temperature increases and as size of sample molecules decreases.

Under fixed chromatographic conditions (same solid phase, mobile phase, flow rate, temperature and detector), the retention times of components remain constant. This property is the key to the success of the chromatographic method. It is used in qualitative analysis to identify the presence of specific components in the sample by comparing the retention times of unknown components to the known standards. The bulk of chromatographic analysis, however, is quantitative. Here peak height and peak area are used to quantitate analytes because they are proportional to the component concentrations. The concentrations of the components analysed must be within linear range of the detector so that the peak height or peak areas of unknown samples can easily be compared to those of

the standard concentrations. Results obtained using peak areas are less affected by the reproducibility of the peak shapes between samples and standards, since the peak area is a function of the peak height and one half peak width for symmetrical peaks. In many instances (18,19) it is impractical to use peak areas, due to unresolved baselines, and peak height is the method of choice. This is especially true when samples have to be extracted from plant or living tissues where coextractants interfere (20). In these cases, it is essential that the peak shape remains constant throughout the analysis of both samples and standards. If the peak broadening occurs and peak height decreases, a significant error in the results can be expected.

D. Methods in HPLC

1. Liquid-Solid or Adsorption Chromatography (LAC)

Adsorption chromatography is the oldest of the four methods. It involves a polar solid stationary phase such as silica or alumina and a non polar or less polar liquid mobile phase, such as pentane or hexane using smaller amounts of a more polar organic modifier, such as dichloromethane or methanol to regulate adsorption properties. Separation occurs when polar solute molecules are adsorbed on to the surface of the stationary phase and are eluted in increasing order of polarity (21). LAC is suitable for samples that are

organic-soluble with intermediate molecular weight of 150-1500 (16) and are nonionic. It is the best method for separating isomeric mixtures.

2. Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) was first used for separation of rare earth, and various fission products in the development of atomic energy. Today it is used in the analysis of protein structures (22), agricultural chemicals (23), drugs (24), trace analysis of compounds in soil and surface water (25), food and food additives (26) and many other substances. IEC involves the use of a stationary phase which has positive or negative charge-bearing functional groups and mobile phase containing the counter ion. The sample ions are in competition with mobile phase ions for the stationary exchange sites. The separations are based on the different strengths of interaction of analytes between the sample ion and the exchange sites.

3. Size Exclusion Chromatography

Size Exclusion Chromatography (SEC), also known as Gel Permeation Chromatography (GPC) is the newest of the four chromatographic methods. It was first introduced by J. C. Moore (27) of Dow Chemical Company in the early 1960s. It is a relatively simple, yet rapid method that can separate high

molecular weight polymers or just simple low molecular weight mixtures. The separation occurs strictly on the basis of molecular size. One of the major differences from other methods is the relative unimportance of solvent type. Here the pore size of the packing has the largest effect on separation characteristics. A change in column packing is comparable to a change in the composition of the mobile phase in other methods. Packings are usually highly porous gels of uniform pore size such as sulfonated polystyrene/divinylbenzene resin, polyvinyl alcohol gels, polyester gels, cross-linked dimethylbenzene-polystyrene gels, and porous silica microspheres which have shown highest performance up to date.

4. Partition Chromatography on Bonded Stationary Phases

Partition Chromatography, often quoted as liquid-liquid chromatography, involves a separation of analytes between two immiscible liquids. One liquid is a mobile phase and another liquid which is usually covalently bonded on to the silica gel surface by siloxane bridges as a stationary phase. The process is similar to the solvent partition between two immiscible liquids in a separatory funnel, except that this method is much faster and more efficient. By selecting an appropriate pair of partitioning liquids almost infinite separation capability can be achieved.

1) Normal-Phase Chromatography

A chromatographic system known as Normal-Phase Chromatography uses the combination of polar bonded stationary phase and less polar mobile phase. The functional groups in the most frequently used bonded stationary phases are: Cyano, Amino, Diamino, Dimethylamino and Glycol phases. The mobile phase usually consists of a non polar organic solvent such as hexane with a polar modifier such as isopropanol. Since reproducibility of retention values and peak symmetry is significantly better for polar bonded phases than for regular silica gel packing, these phases are slowly replacing classical polar adsorbents such as silica and alumina.

2) Reversed-Phase Chromatography

Howard and Martin (28) first reported the use of a nonpolar liquid stationary phase (paraffin and octane) with a polar mobile phase (methanol-water or methanol-acetone mixtures) in 1950. Since phase polarities were in reverse to the conventional system, this chromatographic method has become known as Reversed-Phase Chromatography (RPC). There was little development in this field however, until Stewart and Perry (29) introduced a true bonded nonpolar phases in 1968. In the past several years, the microparticulate nonpolar bonded phases enjoyed the greatest popularity. Today Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) is the most

widely used analytical technique. It is estimated that around 80% of all analytical separations are carried out using this technique.

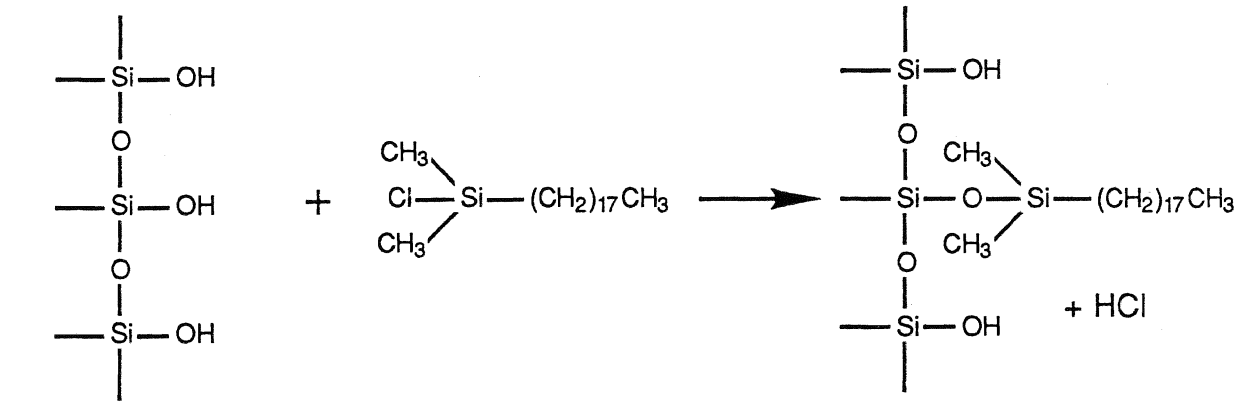
The versatility of this technique is largely due to the broad range of mobile phase compositions. These can range from completely aqueous through any number of aqueous-organic mixtures to completely nonaqueous. Since this branch of chromatography is used in the present study, the factors influencing separation in RP-HPLC will be examined in more detail in the following.

E. Factors Affecting Efficiency of Separation in RP-HPLC

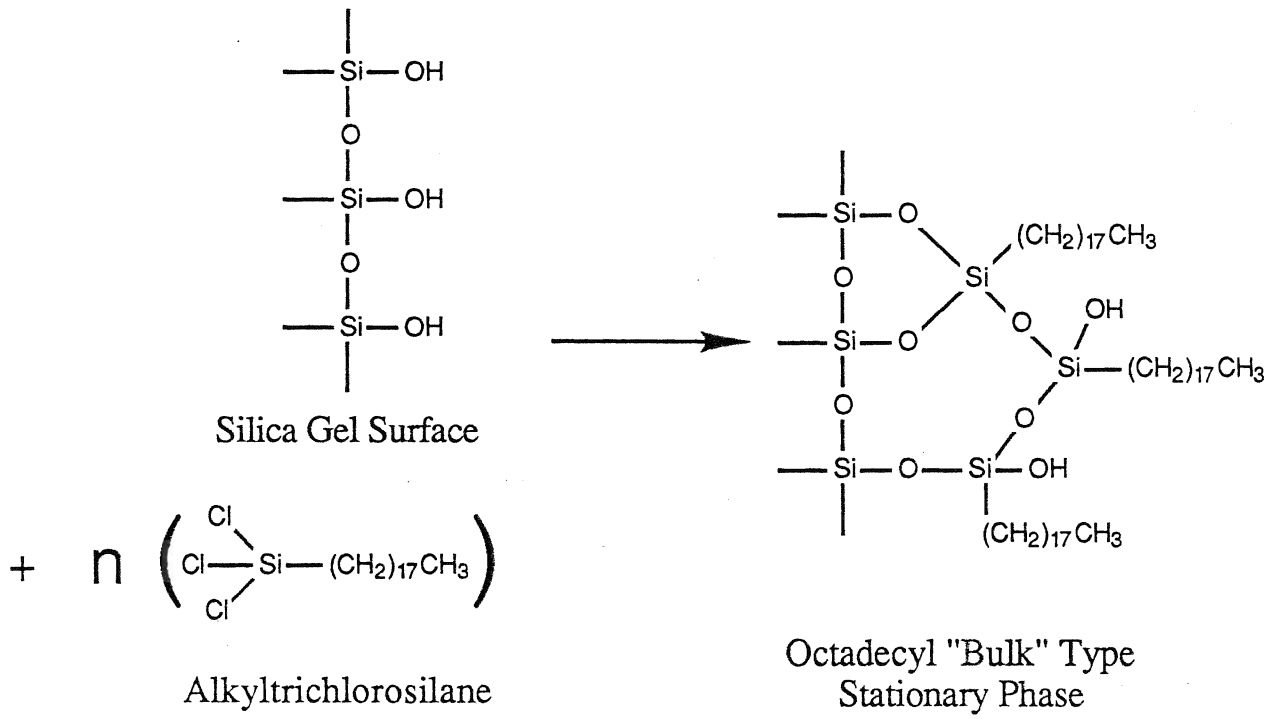
1. Stationary Phase

The stationary phase in RP-HPLC is usually a nonpolar covalently bound hydrocarbonateous octyl, phenyl or octadecyl silica, octadecyl silica being most popular. In section IC it was shown that column separation efficiency increases with a decrease in the particle size of the column packing. At present the most common silica gel particles are in the range of 3-10 μm in diameter and uniform in size. After the proper size of silica gel is selected, it is washed with aqueous acid to generate a high concentration of reactive silanol (SiOH) groups at the surface. The treated silica is then reacted with a silanizing agent as shown in Figure 4. If

FIGURE 4*: Preparation of Hydrocarbonaceous Bonded Phases.



Silica Gel Surface | Alkyldimethylchlorosilane | Octadecyl "Brush" Type Stationary Phase



Silica Gel Surface

Alkyltrichlorosilane

Octadecyl "Bulk" Type Stationary Phase

* from Reference 16.

Table I*

Some Commercially Available RP-HPLC Stationary Phases

Name	Functionality	Base Material	Particle Size (μm)	Description
Partisil ODS-2	Octadecylsilane	Partisil	10	16% Loading
LiChrosorb RP-18	Octadecylsilane	LiChrosorb	5, 10	Monolayer, Stable pH 1-9, 22% Loading
Spherisorb ODS	Octadecylsilane	Spherisorb	5, 10	Spherical, Maximum pH 8, 8% Loading
Zorbax ODS	Octadecylsilane	Zorbax	6-8	15% Loading
LiChrosorb RP-8	Octylsilane	LiChrosorb	5, 10	Monolayer, Stable pH 1-9, 13-14% Loading

*from Reference 16

alkyldimethylchlorosilane is used in silanization, a nonpolar covalently bound "brush" is formed on the surface (30). If the silanizing agent has three reactive groups such as trichlorosilane, the product is a nonpolar crosslinked alkylpolysiloxane or bulk modified layer (31) which enhances coverage of surface silanol groups as shown in Fiure 4. It is impossible to derivatize all the silanol groups due primarily to the steric hindrance from large R groups. It has been shown that more than half of all initially present surface silanols remain underivatized (32).

The percentage of free silanol groups determines the chromatographic properties of the particular stationary phase. It has been shown (33) that these residual silanol groups exhibit strong influence on the chromatographic behavior of polar and hydrogen bonded solutes. A reduction in the number of surface silanols is possible by reaction with trimethylchlorosilane. This process is known as endcapping and was shown to enhance stability of the stationary phase (34). Bij et al. (35) reported that uncapped stationary phases exhibit higher selectivity in some separations. Table I lists some properties of alkyl-silica bonded phases.

There are a large number of commercially available nonpolar bonded phases. Each having somewhat different retention properties which depend on a) Surface area and pore size distribution of the silica; b) Chemical nature of the bonded hydrocarbonaceous moiety; c) Carbon loading on the stationary phase; and d) Surface concentration of accessible

silanol groups. Silica based, nonpolar bonded stationary phases give optimum performance in the pH range from 2-8. In basic eluents, hydrolysis of the silica matrix occurs, which can result in the loss of some organic ligands. In recent years, packings from C18-derivatized polystyrene-divinylbenzene polymer have been introduced. These packings claim high stability and very good reproducibility in the pH range from 0-14 (36).

There is still a lack of complete understanding of the mechanism of solute retention in reverse phase liquid chromatography (37,38). The main reason for this is incomplete understanding of the physical nature of bonded stationary phases and their interaction with the mobile phase and solute molecules. A significant number of papers have been published elucidating retention mechanisms on nonpolar bonded phases. Present understanding is that there are actually three retention mechanisms (39), the primary mechanism being the partition between two liquids, the stationary and mobile phase. The second mechanism suggested by Horvath and Melander (40,41) involves an adsorption mechanism where solute molecules are adsorbed onto the nonpolar stationary phase by Van der Waals dispersion forces. The more polar solutes have a stronger interaction with the polar mobile phase and elute faster. Scott and Kucera (42) proposed that the organic modifier in the mobile phase is preferentially adsorbed onto the bonded phase forming a monolayer with which the solute molecules interact. In this case, the stationary phase serves

only as a support. Scott and Simpson (43) have further shown that the desorption-adsorption coefficients decrease and the distribution coefficients between water and the reverse phase increase exponentially with the carbon number of the moderator. This suggests that the surface of the reverse phase is significantly altered in the presence of the organic modifier. They also found that the dispersive interactions between the reverse phase and the solute hydrocarbon chain are independent of the solute functional groups.

Scott and Simpson (44) studied retention characteristics of "brush" and "bulk"-type reversed phase column packings. They found that the "brush" type reverse phase packings can undergo internal association at low organic modifier concentrations and minimize the effective surface area, resulting in lower retention times than expected. These type of phases need a significant amount of time to equilibrate at low modifier concentrations. The "bulk"-type bonded phases do not exhibit dispersive interactions, equilibrate much faster and exhibit the expected retention behavior.

Tanaka et al.(45) found that the planar solutes were preferentially retained by the planar stationary groups such as octadecyl or large aromatic rings, while aromatic stationary phases showed preferential retention for aromatic and polar solutes.

Stahalberg and Almgren (37) studied the effects of mobile phase composition on the polarity of reverse phases. They found that with increasing concentration of methanol in

the solvent there was a decrease in the polarity of the RP-2 and RP-18 surfaces. This decrease in the polarity of the reverse stationary phase was explained by preferential adsorption of methanol molecules to the free silanol groups through hydrogen bonding. In this way nonpolar methyl groups are exposed to the solute and mobile phase interactions. They also observed that acetonitrile-water mixtures do not behave in the same manner, but that the surface in contact with acetonitrile-water mixtures shows an increase in polarity. This was explained by two processes: the adsorption of acetonitrile through hydrogen bonding which results in a decrease in polarity, as in the case of methanol; in addition, freely moving acetonitrile molecules enter between alkyl chains resulting in an increase in the polarity of the surface layer.

2. Temperature

Most separations at present are carried out at constant, most often room temperature. With the introduction of microbore columns (46) there is more interest in temperature programming which would increase efficiency and decrease retention time.

Melander and coworkers (47) found that retention decreases and efficiency increases with an increase in temperature. This can be explained by the reduction in mobile phase viscosity and faster equilibration between the

stationary phase and mobile phase. Melander et al.(59) also found that in buffered solutions where the pH is close to the pKa of the buffer and the eluate, Van't Hoff plots of the retention factors can deviate from linearity and in some cases can increase with temperature.

3. Mobile Phase

As mentioned earlier the ability to control selectivity by choosing from an infinite number of mobile phase compositions makes RP-HPLC such an attractive analytical technique. Here one changes the mobile phase composition as one changes the column packing in GC analysis to achieve the desired separation. The desired properties of the eluent are low viscosity, high optical transparency at low wavelengths and the appropriate eluotropic strength. In the reverse phase chromatography, the most polar solvent (water) is the weakest eluent, and eluent strength increases as polarity of the solvent decreases. Table II lists properties of some solvents used in chromatography. Acetonitrile, methanol and tetrahydrofuran are most popular organic solvents due to their low viscosity and high UV transparency (48). Four solvent characteristics that contribute the most to the selectivity of the eluate-eluant system are: solvent polarity or chromatographic strength, proton acceptor and proton donor character, and strong dipole character (49).

For most analyses, eluents stronger than water and

Table II*

Properties of Common Organic Solvents
Used as Modifiers in RP-HPLC

	m.w.	B.P.(°C)	ρ^{a}(gcm^{-3})	η^{b}(cP)	ε^{c}	μ^{d}(Debye)	γ^{e}(dyncm^{-1})
Acetonitrile	41.0	82	0.782	0.358	38.80	3.37	29.0
Ethanol	46.1	78	0.789	1.190	24.50	1.68	22.0
Methanol	32.0	65	0.792	0.584	32.70	1.66	22.0
Tetrahydrofuran	72.1	66	0.889	0.510	7.58	1.70	27.6
Water	18.0	100	0.998	1.000	78.50	1.84	73.0

[a] Density at 20°C
[b] Viscosity at 20°C
[c] Dielectric Constant
[d] Dipole Moment
[e] Surface Tension

*Values obtained from Reference 30(p.166)

weaker than pure organic solvent are desired. This is obtained by mixing various amounts of water with the organic solvent. Another way of achieving desired eluent strength, and at the same time reduce retention is by the use of gradient elution. This is achieved by the use of two solvent reservoirs and by increasing the percentage of organic modifier as the analysis progresses. Schoenmakers et al.(50) studied the relationship between retention volume and solvent composition in a linear gradient. Ternary and quaternary mixtures have found some use, due to highly stable pumping systems (51), but still the most popular solvent delivery system is isocratic, and most studies concerning eluent influence are carried out under isocratic conditions.

The effects of mobile phase composition on the selectivity, retention properties and peak shape have been studied extensively (50,52,53,54). Schoenmakers et al.(50) found quadratic relationship between the logarithm of the capacity factor and the volume fraction of organic midifier eq.[8] for mobile phases containing less than 90% water. Their study involved full range of compositions for methanol, acetonitrile and tetrahydrofuran.

$$\ln Ki = AQ^2 + BQ + C \qquad \text{eq.[8]}$$

Q = organic modifier fraction

Karger et al.(54) found that the methylene group selectivity is a linear function of the solvent composition. For methanol-water mixtures selectivity decreases with a decrease in water content of the mobile phase. For acetonitrile-water

Table III*

The Apparent pK* Values of $H_2PO_4^-$ and $HPO_4^=$ for Methanol-Water Mixtures

[Methanol] %v/v	pk* $H_2PO_4^-$	pK* $HPO_4^=$
0.00	2.2	6.9
19.01	2.3	7.1
38.02	2.4	7.3
57.03	2.8	7.9
76.04	3.6	8.8
88.72	4.9	9.6

*data taken from ref.(57)

mixtures, a linear trend is observed up to ca. 40 percent of the acetonitrile composition. Tanaka et al.(55) found that different organic modifiers exhibit significantly different selectivities for aromatic solutes and that the increasing order of selectivity for different organic modifiers corresponds to the order of decreasing eluent strength.

Another very popular method of modifying the aqueous component of the mobile phase is through the use of buffering agents. Buffers are essential for controlling selectivity in ionizable eluates which include most biological molecules. The degree of ionization of weak electrolytes is strongly affected by the mobile phase pH in the area of their pKa. The plot of retention factor k' v. pH of the mobile phase is a sigmoidal curve with the midpoint pH value corresponding to the pKa of the acid (56). Ionization of the eluent usually results in shorter retention times. Buffers can also interact with surface silanols and in this way affect retention or peak shape of nonionizable substances, or they can interact with solute molecules themselves.

The ideal buffer should have uniform buffering capacity in the pH range 2-8, be optically transparent down to 200 nm, be soluble in organic solvents, have potential for masking silanol groups of the stationary phase, and be able to accelerate the rates of protonic equilibria. The alkali-phosphate buffers have most of the desired properties and are extensively used in RP-HPLC. Leitold and Vigh (57)

reported pK of $HPO_4^=$ and $H_2PO_4^-$ in various methanol-water mixtures. They found that as percentage of methanol increases the pK of both salts increases as illustrated in Table III.

Ishimitsu and Hirose (58) studied the effects of pH on the retention behavior of catecol amino-acids using phosphate buffers in the mobile phase. They found that the capacity factor k' depends on the type of solute functional group, and on their pKa as well as on the pH of the eluent. The compounds with pKa higher than 6.0 were independent of the pH of the eluent up to pH of 5.5 and eluted in order of decreasing polarity as expected for true partition chromatography. The ∝ -hydroxy carboxylic acid derivatives exhibit shorter retention as the pH increases from 2.6-5.5 due to increased ionization. A marked increase in retention for some compounds was observed when the pH of the mobile phase was raised from 4.5-8.5. This was attributed to the increase in availability of phosphate anionic species that can form ion-pairs with these compounds.

As quoted before Melander et al. (59) found that in buffered solutions Van't Hoff plots of the retention factors can deviate from linearity, and in some cases retention can increase with temperature. They attributed this to complex formation between sample molecules and buffer species and to masking of the silanol groups of the stationary phase with buffer molecules.

Pepp and Vigh (60) investigated retention behavior of low, medium and high pK aromatic amines using LiChrosorb RP-18

column and acidic aqueous methanol eluents. The eluents, at constant pH, also contained varying concentrations of sodium, potassium and tetra-methyl ammonium salts. They found that salt addition decreased the retention time of protonated amines and to a smaller extent that of the polar, non-ionic solutes. They attributed this trend to the ion exchange of the protonated amine with the dissociated silanols of the RP-18 stationary phase.

It is an accepted fact that the main use of buffers is to maintain the pH of the solution constant, and to serve as background eloctrolytes, but in RP-HPLC proper selection of buffers is essential since buffers can not only enhance resolution but can also cause poor efficiency and assymetric peaks.

4. Sample Solvent

The effects of sample solvent composition in RP-HPLC are still poorly understood. It has been shown by several groups (61-67) that the chromatographic efficiency in RP-HPLC is sensitive to the injection conditions. Two major factors are volume of the injection and the composition of the sample solvent.

Lawerence (61) suggested that the sample solution should contain a slightly larger percentage of water than the mobile phase. This slight excess of water will cause solute molecules

to collect at the head of the column in a narrow band, while sample solution passes onto the column. He observed that the injection of solutes in 100% methanol, acetonitrile or other organic solvents can significantly reduce chromatographic efficiency if mobile phase has large percentage of water. The reason for this is that organic solvents tend to wash the solute down the column at the faster rate than does more polar mobile phase.

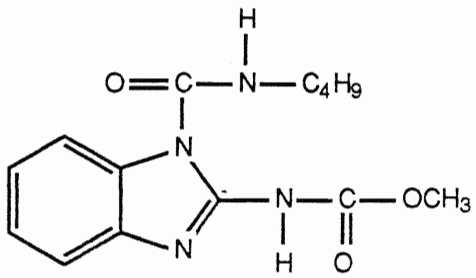
Snyder and Kirkland (62) reported that the sample size should be less than the linear capacity of the column. As the sample size reaches the critical size a noticeable decrease in retention time and resolution is observed. Tsimidou and Macrae (63) observed a significant decrease in resolution as injection volume increased. For packed columns, injection volumes used could vary from 5 to 100 µL (64).

F. Scope of My Research

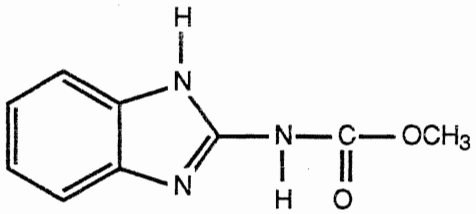
As reported previously, there are well documented studies on the influence of mobile phase composition on the resulting chromatogram. In RP-HPLC the organic modifier composition significantly influences retention time of eluite and changes in the pH of the eluant can often modify the retention of some substances dramatically. It was also shown (60) that the ionic strength influences retention.

Very little has been written about influences of the injection solvent composition on the resulting chromatogram.

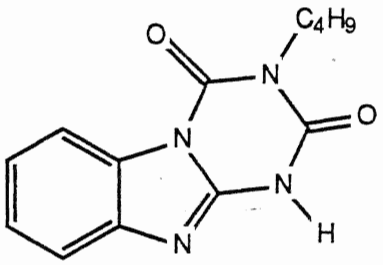
FIGURE 5: Structures of compounds studied



I. Benomyl



II. MBC



III. STB

Most texts advise the use of a solvent composition as in the mobile phase, but this is very often impractical or impossible due to different extraction procedures, different solubilities of the components being analysed, or just inconvenience of dilution. As the columns become smaller, the injection volume will become more important since detector sensitivity will limit further reduction in the injection volume and overloading factors will become more important.

Chiba and Singh (65) in their study of fungicide Benomyl (I), and its degradation products methyl 2-benzimidazolecarbamate (MBC) (II) and 3-butyl-2,4-dioxo[1,2-a]-s-triazinobenzimidazole (STB) (III), observed that the composition of the injection solvent can significantly affect the retention time and the peak shape of the resulting chromatograms. Since the major objective of their studies was not to investigate the systematic effects of injection solvent, it was decided that a separate study should be carried out on the influence of injection solvents in RP-HPLC of these compounds. It was especially interesting since very few published papers (66,67) could be found dealing with this topic. It was felt that this lack of published papers indicated a lack of understanding of the importance of this topic. This disregard for the injection sample composition can be seen currently in the literature where sample solvent composition is almost never mentioned, and even injection volume is excluded in many instances. It was felt that understanding the effects of injection solvent

composition on the peak profiles is very important in quantitative work. This is especially true if an internal standard is used which could be affected to a different degree than the compound of interest.

Tseng and Rogers (66) reported that dihydroxybenzene isomers dissolved in methanol and eluted with water result in broad peaks, shoulders or even split peaks. They attributed these effects to the incomplete mixing of the mobile phase and the injection solvent.

Williams et al.(67) observed that the substantial improvements in the chromatographic performance may be achieved by optimization of the injection solvent composition. They observed significant decreases in the peak height when acetonitrile content in the injection solvent exceeded that of the mobile phase. The same solutes in methanol-water mixtures showed a peak maximum around 30 % methanol. It was also observed by this group that solutes with a high capacity factor exhibit a lesser degree of peak broadening. Both of these groups had no theoretical explanation for these occurences. It was hoped that from our study we could elucidate a mechanism to account for these effects.

My study started in 1982 but due to the circumstances of being a part-time study, it took several years to complete. During this time Tsimidou et al. published two papers (63,68) on the influences of injection solvent composition in the reversed-phase chromatograpy of triglycerides. They examined effects of injection solvent type, polarity of the binary

mixtures and the injection volume on the peak shape of trilaurin and olive oil.

Ng and Ng (69) reported the effects of injection solvent composition on the peak shape of caffeine and salicylamide. By using computer simulated chromatography to reproduce peak distortions, they suggested two main reasons for these distortions. One is a difference in retention ratios between the injected solvent and the mobile phase, and the other is a change in the retention capacity of the column due to the adsorption of the injection solvent onto the column.

Perlman and Kirschbaum (70) studied the effects of ethanol, methanol and water compositions in the injection solvent on a series of compounds. They observed that only compounds capable of forming intramolecular hydrogen bonding exhibited decrease in peak heights with an increase in organic solvent.

Nilsson and Westerland (71) found that the type and the concentration of organic anions present in the injection solvent can have significant influence on the chromatographic efficiency of several benzamides. They observed that as the concentration of organic anion increased peak sharpening occured for some benzamides, while for others it had no effect. They explained this by formation of depletion zones due to replacement of organic modifier on the surface of stationary phase with the organic anion from the injection solvent.

Kirschbaum and Perlman (72) observed split peaks when hydrochlorothiazide was dissolved in methanol and 50 µL of the

solution was injected. When the same compound was dissolved in the mobile phase solvent and 200 µL of the solution was injected, a normal singlet peak was obtained. The mobile phase used in this case was water-methanol-phosphoric acid (75:25:0.02). The reason for splitting is injection of sample in the solvent stronger than mobile phase. This group also observed that peak height for captopril injected in methanol-water mixtures increased with an increase in water content. Their explanation for this is formation of intermolecular and intramolecular hydrogen bonding in methanol which could not exist in aqueous solutions.

Several groups (73-76) have reported presence of anomalous peaks. They were expressed differently as ghost peaks (74), vacant peaks (75) or system peaks (76). In each of the above cases the injection solvent was significantly different from the mobile phase composition.

Rouchouse et al. (77) studied the effects of the sample solvent and the injection volume on the efficiency of columns with 3-µm packings. They found that the efficency is a function of the sample solvent composition and the injection volume.

However, none of the authors discussed the sample solvents which were buffered.

In this study, the effects of six factors in the sample solvent composition were studied on the retention time, peak heigth, peak width, peak area and peak symmetry. The effects studied were:

1. Effect of acetonitrile percentage in the range of 5-50 %.
2. Effect of methanol percentage in the range of 5-50 %.
3. Effect of pH in the range of pH 5-8.
4. Effect of buffer concentrations in the range of 0-0.12 M.
5. Effect of injection volume at constant mass from 10 to 200 µL.
6. Effect of injection volume at increasing mass from 10 to 200 µL.

II. Experimental

A. Instruments

Two HPLC systems were used for chromatographic analyses. One was a Perkin-Elmer (PE) Series 3 High Performance Liquid Chromatograph which was equipped with a Rheodyne constant volume sample-loop injector and a Perkin-Elmer LC-55-S UV detector. Another one was a Hewlett-Packard HP-1090 LC System which was equipped with a HP-79835A solvent delivery system with the flow stability of better than 1%, a HP-79846A autoinjection module which has a programmable injection capability in the volume range from 1 to 250 µL using a syringe-loop injector and an HP-1040A diode array spectrophotometric detector. The latter was also equipped with an HP-85B personal computer. The HP-1040A diode array spectometric detector is capable of generating chromatographic signal (absorbance vs time) used in quantitation of a compound, and a spectrum of a compound (absorbance vs wavelength) which provides qualitative structural information.

A Regis Hi-Chrom reversible column, 5-µm Sperisorb ODS (C-18) 15 cm x 4.6 mm (i.d.) was used throughout the study. A precolumn [5 cm x 4.6mm (i.d.)], dry packed with CO PELL ODS 37-40 µm (Whatman), was used along with the analytical column.

An Accumet(R) pH meter model 810 from Allied Fisher Scientific equipped with a Fisher combination electrode, and a

Metrohm 632 pH meter from Brinkmann Sci., equipped with a wide range Mtrohm combination electrode, were used for pH measurements. Electrodes were standardized with pH 6.96 and pH 4.01 buffers. The pH precision was +/- 0.02 pH units.

For measurements of UV absorbance of the sample solutions before HPLC analysis, a Varian DMS 100 UV-Visible Spectrophotometer was used. Time constant was 0.3 sec., slit width was 1.0, absorbance full sacale was 0-1.0 AU, and scanning range was 340 to 240 nm. This instrument is capable of wavelength accuracy of better than 0.4 nm and wavelength reproducibility of better than 0.2 nm.

Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker AC 200 NMR. Electron impact (EI) and fast atom bombardment (FAB) mass spectra were obtained on a Kratos (AEI) MS-30 double beam, double focusing mass spectrometer, retrofitted with a Kratos FAB source.

B. Materials

1.Compounds Studied: Two degradation products of the fungicide benomyl (I), methyl 2-benzimidazolecarbamate (MBC) (II) and 3-butyl-2,4-dioxo[1,2-a]-s- triazinobenzimidazole (STB) (III) were studied (structures are shown in Figure 5). MBC and STB of analytical grade purity were prepared at Vineland Research Station from Benlate 50% WP as described in (78) and (79) respectively. Purity was confirmed by HPLC (65), Proton NMR and Mass spectral analyses.

2.Solvents: Acetonitrile and methanol used for preparation of sample solutions and as part of the HPLC mobile phase were HPLC grade from Caledon Laboratories Ltd., Georgetown, Ontario.

3.Chemicals: Disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) used in buffer preparation were of reagent grade purity from BDH Chemicals Ltd., Poole, England.

4.Buffer solutions: Buffer solutions for use in the mobile phase and in the sample solvent were prepared by adding individually prepared solutions of Na_2HPO_4 and KH_2PO_4 of 0.07M concentration in a 3:2 (v/v) ratio to obtain final buffer pH of 7.00+/- 0.02.

Buffer solutions used in the study on buffer strengths were prepared as follows: 1.2M buffer by dissolving 0.72 moles of Na_2HPO_4 and 0.48 moles of KH_2PO_4 to one Liter with glass distilled water, 0.70M buffer by mixing 0.70M individually prepared solutions of Na_2HPO_4 and KH_2PO_4 in a ratio 3:2 (v/v) to obtain pH of 7.00+/-0.02. Buffers of 0.07M and 0.007M concentrations were prepared by successive dilutions of 0.7M buffer at pH 7.00+/- 0.02.

Buffer solutions for the pH study were prepared by adding 0.07M individually prepared solutions of Na_2HPO_4 and KH_2PO_4 in suitable proportions to obtain pH 5.00+/-0.02, 6.00+/-0.02, 7.00+/-0.02 and 8.00+/-0.02.

5.Stock Solutions: Stock solutions of the analytes MBC and STB used in the preparation of sample solutions were prepared as

follows:

100 µg/mL STB stock solution was prepared by dissolving 25 mg of STB in 250 mL of acetonitrile. The 20 µg/mL, 10 µg/mL and 5 µg/mL stock solutions were prepared by diluting 20 mL, 10 mL, and 5 mL of the 100 µg/mL STB stock solution respectively to 100 mL with acetonitrile.

100 µg/mL MBC stock solution was prepared by dissolving 25 mg of MBC in 250 mL of methanol. The 20µg/mL, 10µg/mL, and 5 µg/mL stock solutions were prepared by diluting 20 mL, 10 mL, and 5 mL of the 100 µg/mL MBC stock solution respectively to 100 mL with methanol.

C. HPLC Experiments

1. General Procedure

For each factor studied, sample solutions of varying compositions were prepared in duplicate in 100 mL volumetric flasks. Sample solutions (detailed methods of sample preparation are outlined in C4) were injected onto the column (operating conditions are outlined in C5), and results were analysed by measuring retention time, peak height, peak width, peak area and peak symmetry as criteria, as outlined in C6. The major part of the study was carried out on the HP-HPLC unit. For analysis of each series of samples, sample solutions were injected using four different volumes of 10, 50, 100, and

200 µL on the HP-HPLC. Four additional studies were performed on the PE-HPLC unit. They included the studies on the influence of percentage of acetonitrile and methanol in the range from 5 to 30%, study on the influence of pH increase at 0.06M buffer, and the study on the influence of buffer strengths while maintaining 35% acetonitrile. A detailed list of the remaining PE-HPLC studies is shown in the Appendix I.

2. Composition of the Sample Solutions

The basic composition of the sample solutions in this study was 5 µg/mL STB, 5 µg/mL MBC, 5% CH_3CN, 5% CH_3OH, 10% of 0.07 M Phosphate buffer (pH 7), and 80% H_2O. This composition was chosen for two reasons: 1) It was similar to the composition of the mobile phase. The lower percentage of CH_3CN in the sample solvent than in the mobile phase was chosen to allow for the variation in the sample solvent composition for CH_3OH, pH, and buffer strength, without significant influence of a larger percentage of acetonitrile. 2) The presence of CH_3CN was chosen to study the concentration influence of the common organic solvent that is the same as in the mobile phase.

The purpose of CH_3OH was also two fold. It was chosen to study the influence of the most widely used organic solvent that is different from the mobile phase solvent, and secondly, it was used in the preparation of MBC stock solutions.

The concentration of buffer was chosen to approximate

mobile phase composition and to study how buffer concentrations and therefore ionic strength, and pH affect chromatographic performance. It was also of interest to compare the sample solvent effects to those observed by Chiba and Singh (65) and Carbas et al. (80) for the mobile phase.

3. Preliminary Studies to Determine the Optimal Mobile Phase Composition

Optimization of the mobile phase composition was carried out by varying the composition of acetonitrile and buffer strength. Five mobile phase compositions were examined for their separation efficiencies by using resolution as criteria. They were 40% CH_3CN and 10% buffer, 38% CH_3CN and 8% buffer, 35% CH_3CN and 10% buffer, 30% CH_3CN and 10% buffer, and 25% CH_3CN and 5% buffer. The mobile phase consisting of 35:10:55/ CH_3CN-0.07M pH 7 buffer-water v/v, was found to give best performance.

4. Factors Studied for Their Influence on the RP-HPLC Performance

1). Effect of Acetonitrile Concentration in the Sample Solvent

Series 1a(i-vii) solutions varied in acetonitrile concentrations from 5 to 30%. Their buffer concentration was kept constant at 0.06M. Table IVa lists composition of each

Table IVa

Composition of Sample Solutions for Series 1a(i-vii)
in 1) Acetonitrile Concentration Study

Sol'n. No.	%CH_3CN	%CH_3OH	% 1.2M Buffer at pH 7	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	5.0	5.0	5.0	5.0	5.0	85.0
(ii)	10.0	5.0	5.0	5.0	5.0	80.0
(iii)	12.5	5.0	5.0	5.0	5.0	77.5
(iv)	15.0	5.0	5.0	5.0	5.0	75.0
(v)	20.0	5.0	5.0	5.0	5.0	70.0
(vi)	25.0	5.0	5.0	5.0	5.0	65.0
(vii)	30.0	5.0	5.0	5.0	5.0	60.0

Table IVb

Composition of Sample Solutions for Series 1b(i-iv)
in 1) Acetonitrile Concentration Study.

Sol'n. No.	%CH_3CN	%CH_3OH	% 0.07M Buffer at pH 7	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	5.0	5.0	10.0	5.0	5.0	80.0
(ii)	25.0	5.0	10.0	5.0	5.0	60.0
(iii)	35.0	5.0	10.0	5.0	5.0	50.0
(iv)	50.0	5.0	10.0	5.0	5.0	35.0

of the seven solutions. A 50 µL sample of each of these solutions was injected in triplicate to the PE-HPLC and results were analysed. The composition and the chromatographic data for the second series of solutions analysed on the PE-HPLC are shown in Appendix I.

Series 1b solutions were analysed by the HP-HPLC. These solutions varied in acetonitrile concentrations from 5 to 50% with the analyte concentration of 5 µg/mL for MBC and STB. Their buffer concentration was reduced to 0.007M to equal the mobile phase composition and to eliminate the possibility of buffer precipitation at high acetonitrile concentrations. Table IVb lists the complete composition of solutions for series 1b(i-iv). Three more series of solutions 1c(i-iv), 1d(i-iv), and 1e(i-iv), differing only in the concentration of analytes, were prepared. The analyte concentrations were 1µg/mL, 0.5µg/mL, and 0.25 µg/mL, respectively.

2) Effect of Methanol Concentration in the Sample Solution

Six solutions in series 2a(i-vi) were prepared to study the influence of methanol concentration in the sample solvent on the chromatographic performance. These solutions varied in methanol concentrations from 5 to 25%. The buffer concentration in these solutions was 0.06M. The complete list of sample compositions for series 2a is shown in table Va. A 50 µL aliquot of each of the solutions from series 2a was analysed on the PE-HPLC. The composition and the chromatographic data for the second series of solutions

Table Va

Composition of Sample Solutions for Series 2a(i-vi)
in 2) Methanol Concentration Study

Sol'n. No.	%CH_3CN	%CH_3OH	% 1.2M Buffer at pH 7	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	5.0	5.0	5.0	5.0	5.0	85.0
(ii)	5.0	10.0	5.0	5.0	5.0	80.0
(iii)	5.0	12.5	5.0	5.0	5.0	77.5
(iv)	5.0	15.0	5.0	5.0	5.0	75.0
(v)	5.0	20.0	5.0	5.0	5.0	70.0
(vi)	5.0	25.0	5.0	5.0	5.0	65.0

Table Vb

Composition of Sample Solutions for Series 2b(i-iv)
in 2) Methanol Concentration Study.

Sol'n. No.	%CH_3CN	%CH_3OH	% 0.07M Buffer at pH 7	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	5.0	5.0	10.0	5.0	5.0	80.0
(ii)	5.0	25.0	10.0	5.0	5.0	60.0
(iii)	5.0	35.0	10.0	5.0	5.0	50.0
(iv)	5.0	50.0	10.0	5.0	5.0	35.0

analysed on the PE HPLC are listed in Appendix I.

Series 2b(i-iv) samples were analysed on the PE-HPLC. In these solutions methanol concentrations varied from 5 to 50%. In this series the buffer concentration in the sample was the same as for the mobile phase of 0.007M. The compositions of individual solutions for series 2b(i-iv) are listed in Table V b. Series 2c(i-iv), 2d(i-iv), and 2e(i-iv) differed in composition from 2b(i-iv) only in analyte concentrations which were 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL respectively.

3) Effect of pH of the Sample Solution

The solutions in series 3a(i-vii) were prepared to study the influence of pH in the sample solutions on the chromatographic peak profiles of STB and MBC. For this series of solutions the pH of the 1.2M buffer added to each solution varied from pH 4.42 to pH 9.10. This series was analysed on the PE-HPLC. The composition of individual solutions for series 3a(i-vii) are listed in Table VIa. A 50 µL aliquot of each solution was injected. For the second series of solutions analysed on the PE-HPLC, the solution composition and the chromatographic results are shown in Appendix I.

For the analysis on the HP-HPLC, solutions differing only in the analyte concentration were prepared. For series 3b(i-iv), analyte concentrations were 5 µg/mL for both STB and MBC. Complete compositions of series 3b(i-iv) samples are listed in Table VIb. Series 3c(i-iv), 3d(i-iv), and 3e(i-iv) had the same compositions as series 3b(i-iv) except analyte

Table VIa

Composition of Sample Solutions for Series 3a(i-vii)
in 3) pH Study

Sol'n. No.	%CH_3CN	%CH_3OH	% 1.2M Buffer	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	5.0	5.0	5.0 at pH 4.42	5.0	5.0	85.0
(ii)	5.0	5.0	5.0 at pH 5.95	5.0	5.0	85.0
(iii)	5.0	5.0	5.0 at pH 6.50	5.0	5.0	85.0
(iv)	5.0	5.0	5.0 at pH 6.74	5.0	5.0	85.0
(v)	5.0	5.0	5.0 at pH 7.40	5.0	5.0	85.0
(vi)	5.0	5.0	5.0 at pH 7.90	5.0	5.0	85.0
(vii)	5.0	5.0	5.0 at pH 9.10	5.0	5.0	85.0

Table VIb

Composition of Sample Solutions for Series 3b(i-iv)
in 3) pH Study.

Sol'n. No.	%CH_3CN	%CH_3OH	% 0.07M Buffer	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	5.0	5.0	10.0 at pH 5.0	5.0	5.0	80.0
(ii)	5.0	5.0	10.0 at pH 6.0	5.0	5.0	80.0
(iii)	5.0	5.0	10.0 at pH 7.0	5.0	5.0	80.0
(iv)	5.0	5.0	10.0 at pH 8.0	5.0	5.0	80.0

Table VIIa

Composition of Sample Solutions for Series 4a(i-vii)
in 4) Buffer Strength Study

Sol'n. No.	%CH_3CN	%CH_3OH	% Buffer at pH 7.0	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	35.0	5.0	10.0 at 1.400M	5.0	5.0	50.0
(ii)	35.0	5.0	10.0 at 0.700M	5.0	5.0	50.0
(iii)	35.0	5.0	10.0 at 0.070M	5.0	5.0	50.0
(iv)	35.0	5.0	10.0 at 0.007M	5.0	5.0	50.0
(v)	35.0	5.0	no buffer added	5.0	5.0	60.0

Table VIIb

Composition of Sample Solutions for Series 4b(i-iv)
in 4) Buffer Strength Study.

Sol'n. No.	%CH_3CN	%CH_3OH	% Buffer at pH 7.0	[STB] µg/mL	[MBC] µg/mL	%H_2O
(i)	5.0	5.0	10.0 at 1.200M	5.0	5.0	80.0
(ii)	5.0	5.0	10.0 at 0.700M	5.0	5.0	80.0
(iii)	5.0	5.0	10.0 at 0.070M	5.0	5.0	80.0
(iv)	5.0	5.0	10.0 at 0.007M	5.0	5.0	80.0
(v)	5.0	5.0	no buffer added	5.0	5.0	90.0

concentrations were reduced to 1, 0.5, and 0.25 µg/mL respectively.

4) Effect of Buffer Concentration in the Sample Solution

Two series of solutions with varying buffer concentrations, from 0 to 0.14M, were analysed on the PE-HPLC. The composition of series 4a(i-iv) samples is listed in Table VIIa. For the remaining series the composition and the chromatographic data are reported in the Appendix I.

Four series of solutions were analysed on the HP-HPLC. The composition of series 4b(i-iv) samples is reported in Table VIIb. Series 4c(i-iv), 4d(i-iv), and 4e(i-iv) sample solutions varied from 4b only in analyte concentrations, which were 1, 0.5, and 0.25 µg/mL respectively.

5) Influence of Injection Volume at Increasing Mass

Four injection volumes of 10, 50, 100, and 200 µL at analyte concentrations of 5 µg/mL were studied for the b series of samples for each of the above four factors.

6) Influence of Injecton Volume at Constant Mass

Four injection volumes of 10, 50, 100, and 200 µL at analyte concentrations of 5, 1, 0.5, and 0.25 µg/mL were studied for series b, c, d, and e, for each of the four factors.

5. Operating Parameters

1) Mobile Phase : The composition of the mobile phase used throughout the study was CH_3CN-H_2O-pH 7 buffer, (35:55:10) v/v and the system was run isocratically. All mobile phase solutions were filtered through 0.45 um membrane filter and degassed for 5 minutes under a vacuum. During the analysis on HP-HPLC, mobile phase solvents were completely purged with helium.

2) Flow Rate : A mobile phase flow rate of 1 mL/min was used for the entire study.

3) Injection Volume : In PE-HPLC analyses, solutions were introduced through a constant volume loop of 10 or 50 µL. In the HP-HPLC an automatic sampler with variable volume injector was used allowing unattended operation of the instrument. Injection volumes were varied by computer command from 10 to 200 µL. When different concentrations of analytes were involved, analyses were carried out in the increasing order of concentrations to minimize potential errors due to carry over of previously injected samples.

4) Recorder Settings : For PE-HPLC analyses the chart speed was set to 0.5 cm/min and voltage setting was 1 millivolt full scale. For analyses on HP-HPLC chart speed was set to 1 cm/min.

5) Detector Settings : The detection wavelength was 280 nm throughout. For the PE-HPLC detector, the response mode was

normal with FS absorbance of 0.1AU for 50 µL injectons and 0.02AU for 10 µL injections. For the HP instrument absorbance range was 280, 140, 70, and 14 mAU for injection volumes of 200, 100, 50, 10 µL, respectively. The absorbance spectrum from 340 to 240 nm was recorded at the apex of each chromatographic peak to observe possible changes in absorbance profiles due to variations in sample composition.

6) Temperature : Analyses were carried out at room temperature (22-25^{o}C) on the PE instrument and at 40^{o}C on the HP-HPLC.

6. Analysis of Chromatographic Results

1) Criteria Used to Assess the Chromatographic Performance

a. Retention Time : defined as the time taken to reach the apex of the peak, where the apex is the point at which the first derivative changes from positive to negative.

b. Peak Height : defined as the perpendicular distance from the apex to the baseline.

c. Peak Width : defined as a time span between up and down slope of the peak at half height and is calculated from the equation 0.3T + 0.7A/H where T = time between peak inflection points, A = corrected area, and H = corrected height. If inflection point is not found peak width is calculated as A/H.

d. Peak Area : The start of the area count occurs when (1) the first derivative of two consecutive data points is greater than the slope threshold, (2) the second derivative is

positive, and (3) the end of the area count occurs when the first and second derivatives are respectively smaller than the slope and the curvature threshold.

e. Peak Symmetry : calculated from the equation:

$$\text{Symmetry} = (A_1+A_2)/(A_3+A_4) \qquad \text{eq. [9]}$$

where: A_1= area between start of the peak and front inflection point; A_2= area between front inflection point and peak apex; A_3= area between apex and rear inflection point; A_4= area between rear inflection point and end of peak. Above definitions were obtained from HP Operating Manual.

2) Plots

Plots of retention time, peak height, and peak width verses the change in the solution composition were made for each of the factors studied.

D. Additional Experiments

1. pH Measurements

The pH values of the buffer solutions before adding organic solvents and the apparent pH* values of the sample solutions as prepared with the presence of acetonitile and methanol at various compositions were measured. Results are reported in Table XX.

2.UV Absorbance Measurements

To study the possible effects due to the composition changes of the sample solvent on the UV absorbance profiles of STB and MBC, individual solutions containing only STB or MBC were prepared. These solutions were in every other respect same as solutions from C3(1-4). A UV scan of each solution was recorded from 340 to 240 nm. The UV data are shown in Table XXI.

3.Mass Spectral Anlyses

Both compounds were analysed by EI and FAB mass spectral technigue in hope to learn more about their hydrogen bond character.

4. Proton NMR and Infra Red Spectral Analyses

The above analyses were attempted in hope to gain more information on the sample solvent interaction, but due to low solubility of analytes and only in highly polar solvents no useful information was obtained.

E. Linearity of Detector Response and Reproducibility

For MBC, linear response was established for 5 µg/mL

solutions from 10 µL up to 200 µL injection volume with correlation coefficient of 0.99967. For STB at 5 µg/mL response was linear from 10 µL up to 200 µL injection volume with coefficient of correlatrion of 0.99953.

The coefficient of variation in the injection reproducibility on PE-HPLC, for six consecutive injections of solution (i) from series 1a, was 0.6% for retention time and 1.0% for peak heights. For HP-HPLC, the reproducibilty error for 24 injections at 10 µL injection volume for STB in series 1b was 0.1% for retention time, 0.7% for peak height, 0.5% for peak width and 2.5% for peak area.

III. Results

The effects of the sample composition in the RP-HPLC analysis on the peak profiles of two carbamate compounds STB and MBC were examined. These were: effect of methanol increase, effect of acetonitrile increase, change in the pH and increase in buffer concentration. The effects were studied by examining changes in retention times, peak areas, peak heights, peak widths and peak symmetries at varying sample compositions. The effects of injection volume at increasing mass and at constant mass were also studied.

Data from duplicate samples, each injected in triplicate were obtained from the HP-85 computer for HP-HPLC analyses and were obtained manually for PE-HPLC analyses. Data were further reduced by calculating mean and standard deviation for triplicate injections. Results are listed in Tables VIII-XIX, and plots and chromatograms illustrating observed trends are shown in Figures 6-61.

A. Effect of Acetonitrile Concentration in the Sample Solution on the Chromatographic Peak Profile

1.Analysis on PE-HPLC

There was no pronounced influence on the retention time of STB or MBC as acetonitrile concentration was increased from

Table VIII*

Chromatographic Results for STB and MBC when [CH_3CN] Increases from 5 to 30% at 0.06M Buffer Concentration

[CH_3CN]	Retention Time (min)		Peak Height (cm)	
%	STB	MBC	STB	MBC
5.0	3.42±.02	4.48±.02	10.10±.10	9.15±.05
10.0	3.40±.02	4.50±.00	10.60±.10	9.02±.08
12.5	3.40±.00	4.50±.02	10.18±.06	9.03±.05
15.0	3.42±.02	4.50±.02	10.02±.08	8.85±.05
20.0	3.40±.00	4.50±.00	9.85±.07	8.80±.00
25.0	3.40±.00	4.50±.00	9.90±.10	8.82±.10
30.0	3.40±.00	4.48±.02	9.88±.10	8.54±.07

*—50μL injection volume

5 to 30%, at 50 µL injection volume and 0.06M buffer concentration (Table VIII). The peak height, however, decreased by 6.7% for MBC and by 2.3% for STB.

2.Analysis on HP-HPLC

Two major differences from the PE study were: 1) The buffer concentration was reduced from 0.06 to 0.007M to equal that of the mobile phase; 2) The acetonitrile concentration range was increased to 50%, to include the concentration higher than that in the mobile phase of 35%.

1) At Constant Mass of Analyte Injected

Retention Time As the concentration of acetonitrile in the sample solvent increased from 5 to 50%, the retention time of the STB peak decreased (Table IXa). The reduction in retention time was enhanced by an increase in the injection volume. For 10, 50, 100, and 200 µL injections, retention times decreased by 1.9, 6.9, 9.8, and 11.8% respectively. At the same solution composition, the retention times of the analytes increased with the the increase in the injection volume but the increases became less pronounced as the concentration of acetonitrile in the sample solvent became greater, as can be observed in Figures 7. At 5% acetonitrile, retention times were 2.271, 2.366, 2.440, and 2.575 minutes and at 50% acetonitrile, retention times were 2.202, 2.203, 2.20, and 2.271 minutes for 10, 50, 100, and 200 µL injections, respectively.

Table IXa

Chromatographic Results for STB when [CH_3CN] in the Sample Solvent increases from 5 to 50% and Injected Mass of Analytes Stays Constant at Increasing Injection Volume.

CH3CN CONC. %	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10 µL @ 5µg/mL			
5	2.271±.001	77±1	9.84±.08	0.109±.001	0.36±.01
5	2.272±.001	77±1	9.69±.01	0.116±.001	0.37±.01
25	2.255±.003	83±4	9.01±.15	0.128±.001	0.36±.01
25	2.256±.003	81±1	8.95±.01	0.126±.001	0.36±.01
35	2.241±.003	85±4	8.44±.10	0.139±.005	0.35±.01
35	2.241±.002	82±1	8.28±.08	0.138±.004	0.36±.01
50	2.227±.002	76±3	7.21±.06	0.151±.003	0.41±.01
50	2.230±.002	76±1	7.19±.03	0.154±.003	0.42±.01
		50 µL @ 1µg/mL			
5	2.366±.002	89±9	12.47±.02	0.098±.001	0.32±.01
5	2.364±.001	92±6	12.51±.08	0.111±.005	0.31±.02
35	2.274±.001	87±1	8.97±.06	0.139±.001	0.41±.01
35	2.276±.001	87±1	9.12±.04	0.138±.001	0.41±.01
50	2.203±.003	80±2	5.39±.03	0.210±.010	0.58±.01
50	2.206±.001	80±2	5.37±.03	0.219±.009	0.59±.02
		100µL @ .5µg/mL			
5	2.440±.001	90±1	12.57±.07	0.098±.001	0.30±.01
5	2.441±.003	92±3	12.60±.10	0.099±.002	0.29±.01
35	2.319±.002	89±1	8.83 ±.02	0.146±.002	0.99±.01
35	2.319±.002	88±1	8.76 ±.05	0.145±.001	0.99±.01
50*	2.170±.020	—	4.5	—	0.79±.08
50*	2.180±.006	—	4.1	—	0.74±.01
		200µL @ .25µg/mL			
5	2.575±.002	83±1	11.45±.08	0.099±.001	0.29±.01
5	2.575±.002	86±6	11.61±.12	0.101±.003	0.28±.01
35	2.413±.003	80±2	7.28±.05	0.164±.003	0.62±.03
35	2.414±.005	80±1	7.17±.12	0.163±.004	0.63±.01
50	2.271±.009	50±4	2.55±.09	0.251±.046	0.54±.02
50	2.230±.020	—	1.19±.01	—	0.77±.06

Table IXb

Chromatographic Results for MBC when [CH_3CN] in the Sample Solvent increases from 5 to 50% and Injected Mass of Analytes Stays Constant at Increasing Injection Volume.

CH3CN CONC. %	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10 µL @ 5µg/mL			
5	3.323±.003	104±6	9.16±.11	0.163±.010	0.42±.03
5	3.326±.001	98±4	8.92±.19	0.156±.001	0.42±.01
25	3.329±.003	104±1	8.62±.09	0.169±.003	0.40±.02
25	3.332±.003	106±1	8.72±.01	0.170± 0	0.41± 0
35	3.317±.002	107±3	8.60±.08	0.176±.005	0.41±.01
35	3.320±.003	105±2	8.47±.16	0.178±.002	0.40±.01
50	3.316±.004	101±3	8.15±.06	0.183±.007	0.42±.01
50	3.320±.002	104±8	8.46±.61	0.188±.010	0.41±.04
		50 µL @ 1µg/mL			
5	3.340±.002	114±1	11.38±.05	0.143±.001	0.39±.01
5	3.340±.001	113±4	11.34±.07	0.145±.005	0.40±.01
35	3.284±.002	114±3	8.73±.09	0.143±0	0.46±.02
35	3.284±.002	114±1	8.81±.02	0.191±.005	0.45±.01
50	3.251±.001	114±2	7.64±.08	0.210±.010	0.48±.01
50	3.253±.003	109±6	7.43±.22	0.218±.005	0.51±.03
		100µL @ .5µg/mL			
5	3.405±.001	112±3	11.87±.09	0.135±.001	0.38±.01
5	3.404±.001	114±3	11.88±.07	0.136±.004	0.38±.01
35	3.318±.001	114±2	7.89±.02	0.213±.005	0.54±.02
35	3.319±.002	113±1	7.84±.03	0.215±.006	0.53±.01
50	3.265±.003	114±1	5.76±.10	0.281±.019	0.66±.01
50	3.280±.010	113±2	5.73±.03	0.265 ±.012	0.72±.09
		200µL @ .25µg/mL			
5	3.532±.003	117±3	12.21±.05	0.134±.003	0.37±.01
5	3.533±.002	115±3	12.73±.03	0.132±.003	0.39±.01
35	3.388±.007	119±2	6.60±.10	0.290±.030	0.62±.04
35	3.426±.003	115±1	6.30±.10	0.270±.030	0.61±.01
50	3.320±.040	119±3	3.66±.05	0.398±.002	0.67±.06
50	3.290±.010	—	3.59±.07	—	0.65± 0

Figure 6: Chromatograms of STB (1) and MBC (2) for Constant Mass Study Showing the Effects of the Increasing Acetonitrile Concentration in the Sample Solvent. 1) 5% CH_3CN; 2) 35% CH_3CN; 3) 50% CH_3CN.

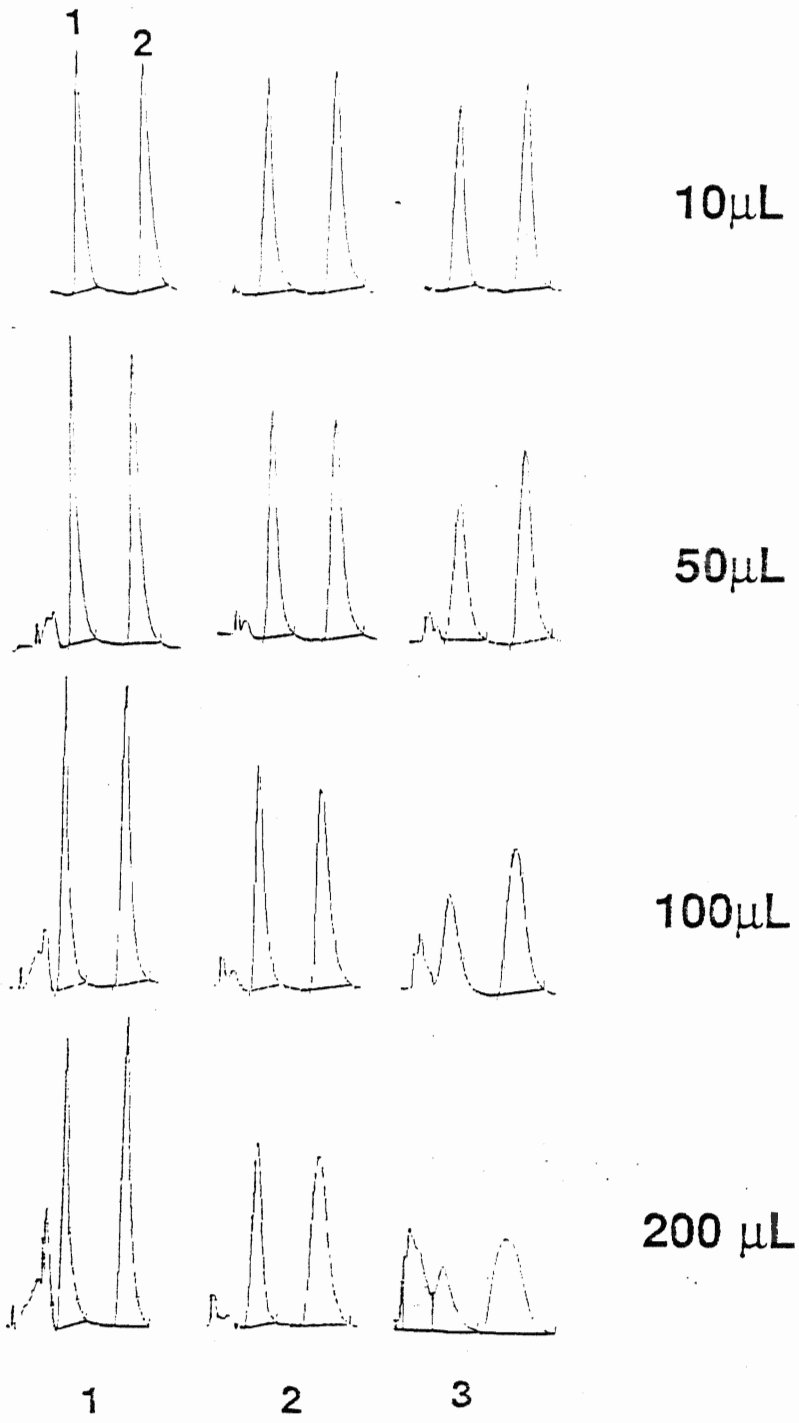


Figure 7: Plot of Retention Time vs. Acetonitrile Concentration in the Sample Solvent for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

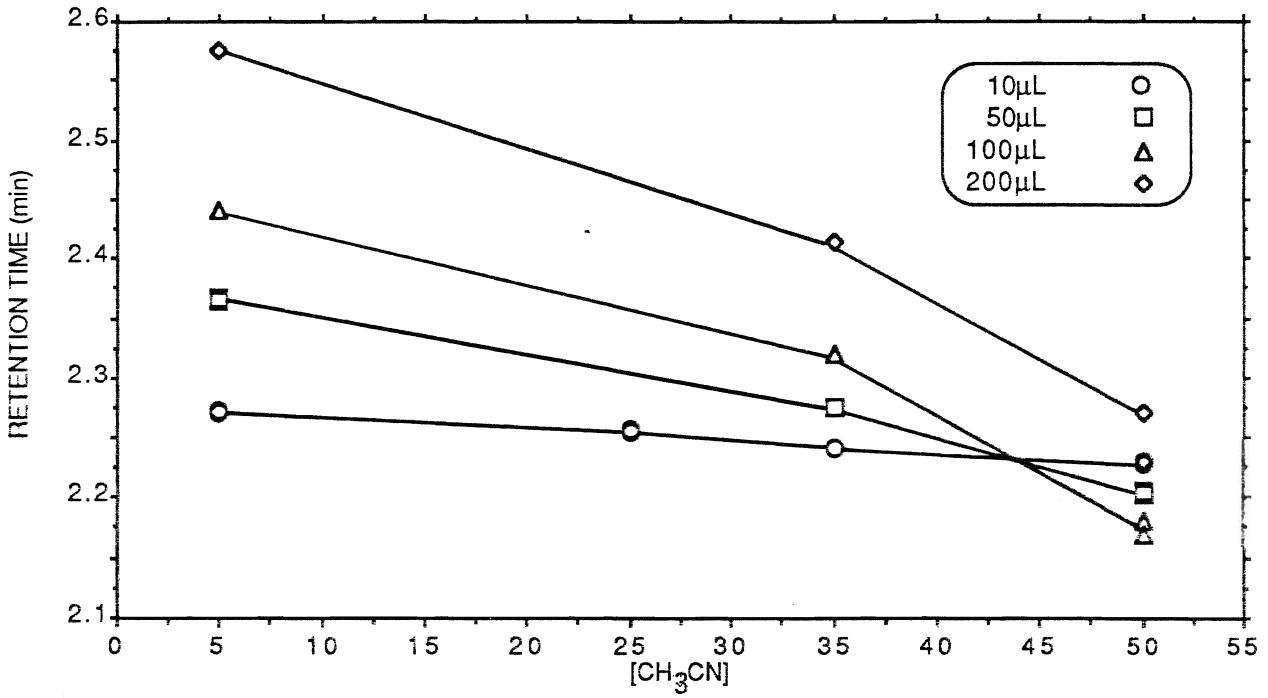


Figure 8: Plot of Retention Time vs. Acetonitrile Concentration in the Sample Solvent for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

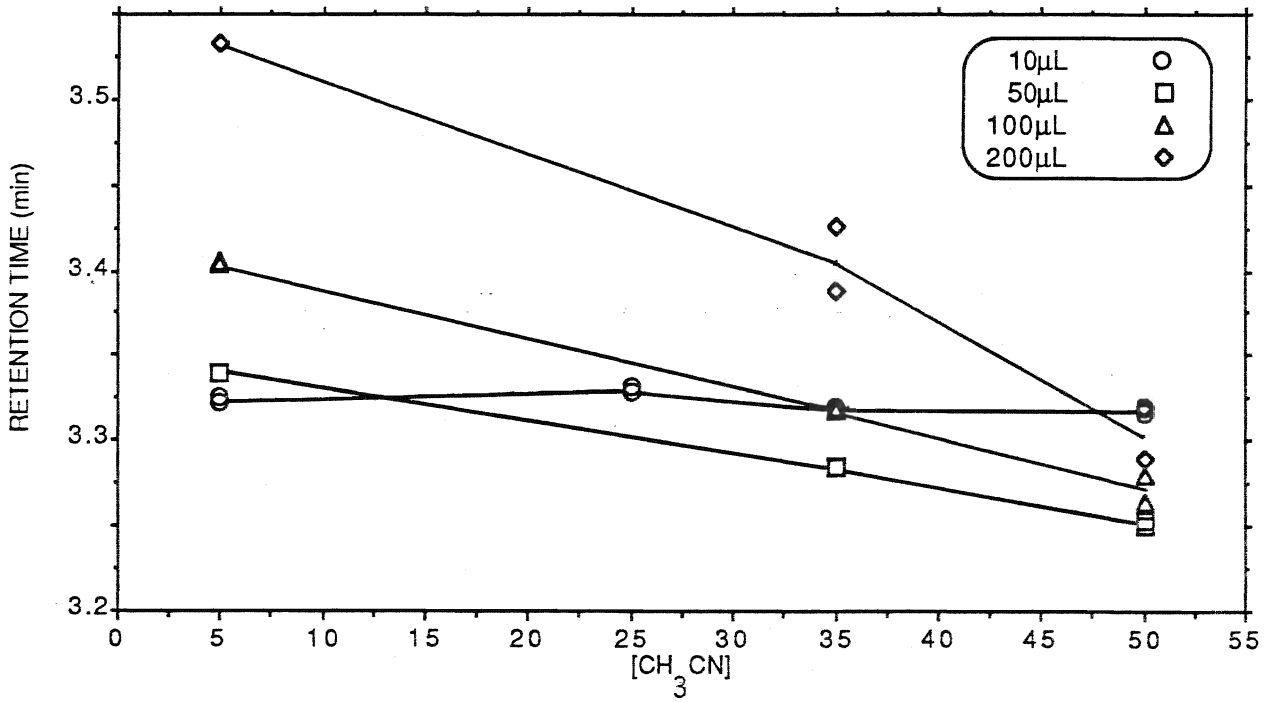


Figure 9: Plot of Peak Height vs. Acetonitrile Concentration in the Sample Solvent for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

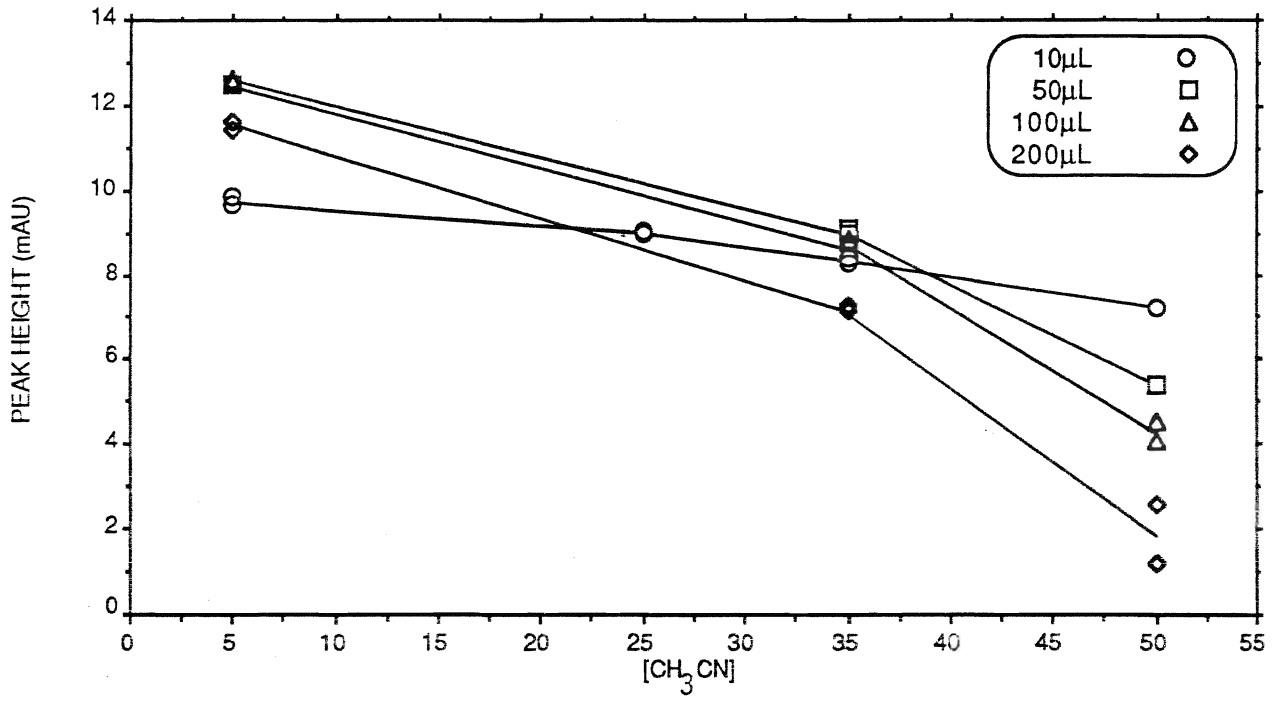


Figure 10: Plot of Peak Height vs. Acetonitrile Concentration in the Sample Solvent for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

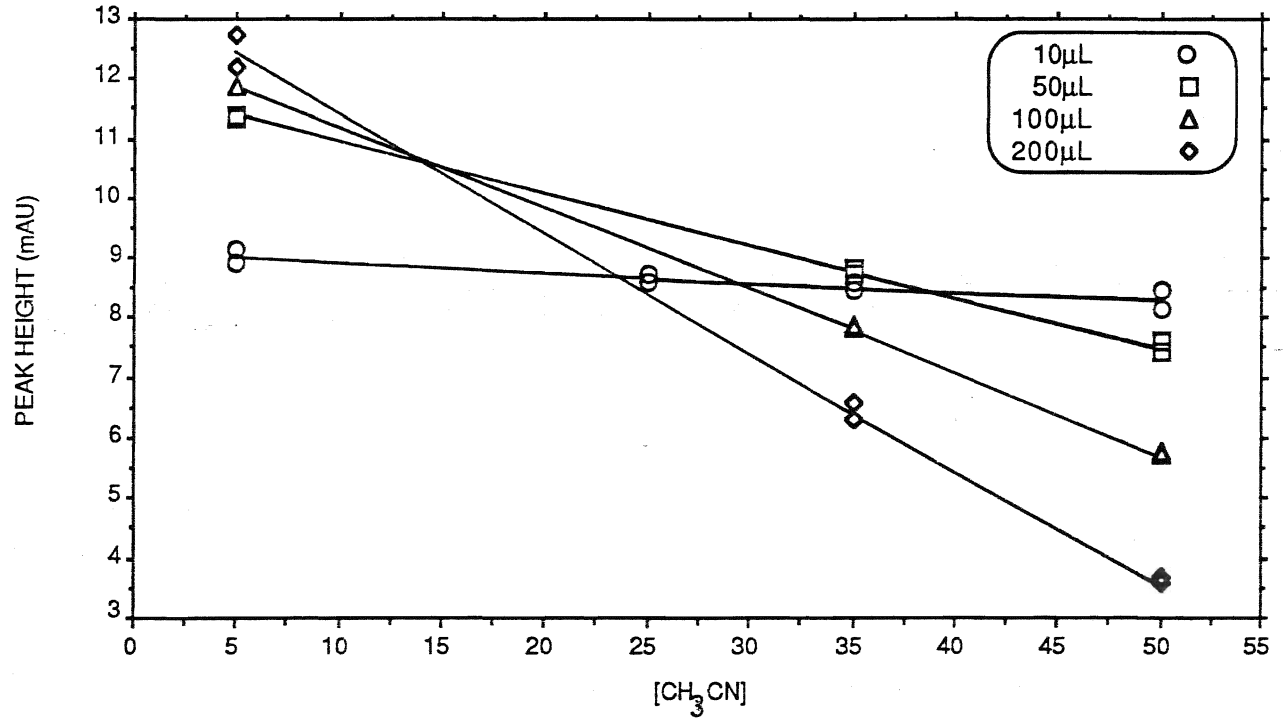


Figure 11: Plot of Peak Width vs. Acetonitrile Concentration in the Sample Solvent for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

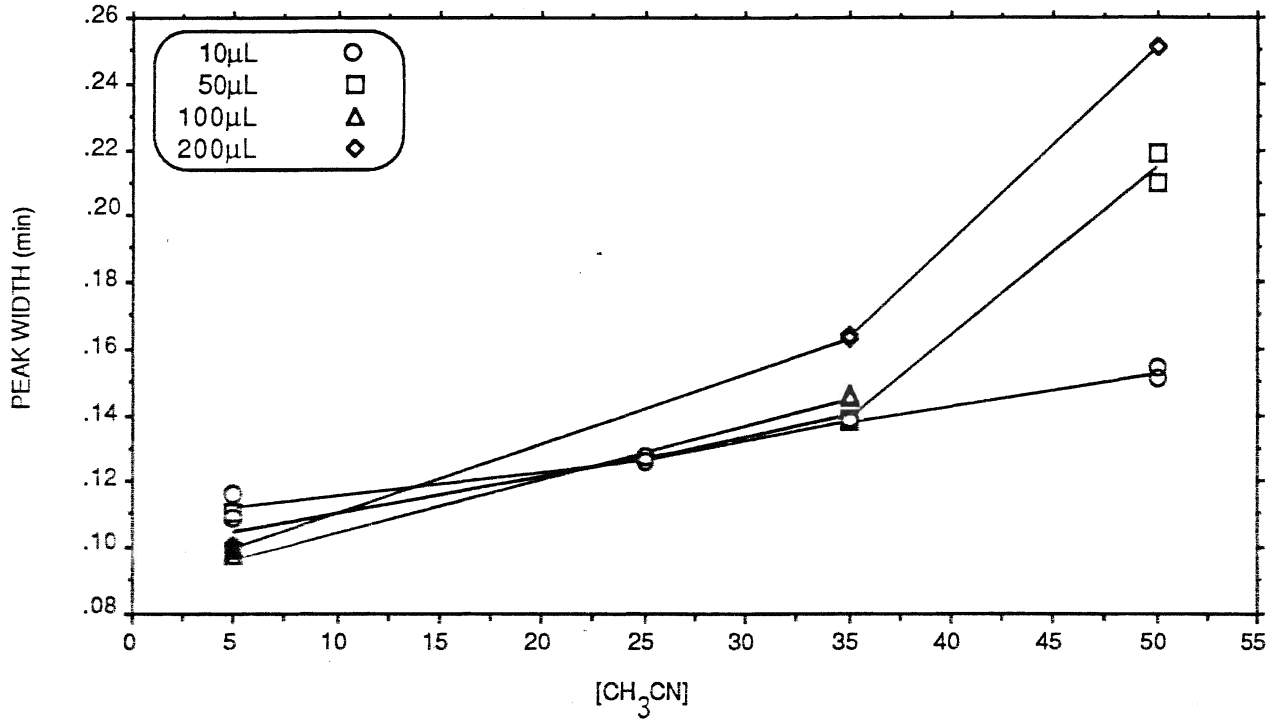
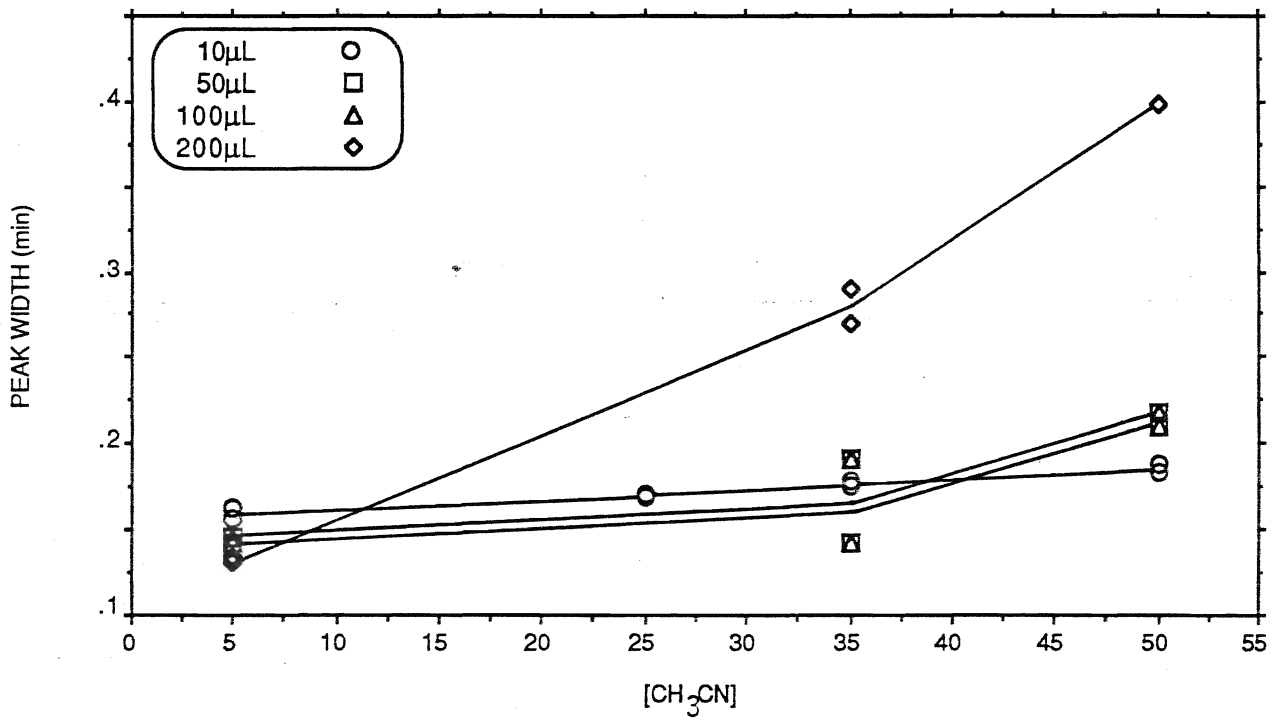


Figure 12: Plot of Peak Width vs. Acetonitrile Concentration in the Sample Solvent for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.



A decreasing trend in the retention time with the increase in the acetonitrile concentration was also observed for MBC but to a somewhat smaller extent (Figure 8). Decreases of 1.0, 2.7, 4.1, and 6.0% were observed for 10, 50, 100 and 200 µL injection volumes. Here again retention time increased as the injection volume increased, for the same solution composition, and the increase became less pronounced as acetontrile concentration increased. Complete results are listed in Table IXb.

Peak Height The increase in the acetonitrile concentration also had strong influence on the peak heights of STB (Figure 9) and MBC (Figure 10). For both analytes peak heights decreased with the acetonitrile increase. The decrease in the peak height was more prominent at larger injection volumes. For STB, peak heights decreased by 26.7, 56.9, 64.2, and 77.7%, while for MBC, decreases were 8.2, 33.7, 51.6, and 70.9% for 10, 50, 100, and 200 µL respectively. The peak height influence was greater for STB than MBC, especially at lower acetonitrile concentrations.

Peak Width The peak widths of both analytes increased with the increase in acetonitrile percentage at all four injection volumes (Tables IXa and IXb). The increases became greater for larger injection volumes as can be observed in Figures 11 and 12. An interesting point was that for STB at 5% acetonitrile, peak widths were the same for all four injection volumes while for MBC at 5% acetonitrile, an actual decrease in the peak width was observed with the increase in the injection volume.

At 50% acetonitrile, however, a significant increase in the peak widths was observed. For MBC at 5% acetonitrile peak widths were 0.162, 0.143, 0.135, and 0.134 minutes, while at 50% acetonitrile peak widths were 0.170, 0.219, 0.281, and 0.398 minutes, for 10, 50, 100, and 200 µL injections, respectively.

Peak Area The peak areas for STB remained constant from 5 to 35% acetonitrile, and decreased for 50% acetonitrile. The decrease became more pronounced as the injection volume increased. A reason for this was that a larger proportion of STB was eluted in the split peak as the injection volume increased. The area of the MBC peak remained constant at all acetonitrile concentrations at all four injection volumes (Table IXa and IXb).

Peak Symmetry A significant increase in the peak symmetry (which is an improvement) was observed for both analytes with the increase in the acetonitrile concentration. The increase was somewhat larger for STB than MBC as can be observed in Tables IXa and IXb.

2) At Increasing Mass of Analyte Injected

Four sets of experiments were performed. For each experiment, anlyte concentrations were constant at 5 µg/mL as the injection volume was increased from 10 to 50, 100, and 200 µL. Complete chromatographic results are listed in Table Xa for STB and Table Xb for MBC. To compare these results to those of the constant mass study, the peak area and the peak

Table Xa

Chromatographic Results for STB when [CH_3CN] in the Sample Solvent increases from 5 to 50% and Injected Mass of Analytes Increases with Increasing Injection Volume.

CH3CN CONC. %	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10 µL @ 5µg/mL			
5	2.271±.001	77±1	9.84±.08	0.109±.001	0.36±.01
5	2.272±.001	77±1	9.69±.01	0.116±.001	0.37±.01
25	2.255±.003	83±4	9.01±.15	0.128±.001	0.36±.01
25	2.256±.003	81±1	8.95±.01	0.126±.001	0.36±.01
35	2.241±.003	85±4	8.44±.10	0.139±.005	0.35±.01
35	2.241±.002	82±1	8.28±.08	0.138±.004	0.36±.01
50	2.227±.002	76±3	7.21±.06	0.151±.003	0.41±.01
50	2.230±.002	76±1	7.19±.03	0.154±.003	0.42±.01
		50 µL @ 5µg/mL			
5	2.378±.002	95±5	12.20±.10	0.106±.004	0.28±.01
5	2.376±.001	95±3	12.05±.08	0.108±.003	0.29±.02
25	2.329±.001	92±1	10.62±.03	0.123±.001	0.35±.01
25	2.326±.001	91±1	10.57±.04	0.119±.004	0.38±.03
35	2.285±.001	92±1	8.68±.05	0.153±.001	0.38±.01
35	2.286±.001	97±3	8.70±.10	0.159±.003	0.36±.03
50	2.224±.001	88±2	5.24±.04	0.249±.003	0.61±.02
50	2.225±.004	89±1	5.30±.06	0.255±.007	0.52±.07
		100µL @ 5µg/mL			
5	2.457±.004	92±1	12.11±.01	0.103±.001	0.28±.01
5	2.458±.002	92±1	11.96±.08	0.104±.001	0.28±.01
25	2.400±.001	94±1	10.77±.03	0.124±.001	0.34±.01
25	2.396±.001	93±2	10.72±.10	0.122±.003	0.35±.01
35	2.335±.002	93±2	8.58±.04	0.157±.002	0.45±.01
35	2.335±.003	93±1	8.40±.01	0.159±.001	0.45±.01
50	2.229±.001	81±2	4.03±.02	0.286±.006	0.62±.01
50	2.244±.009	81±2	4.03±.02	0.289±.001	0.62±.01
		200µL @ 5µg/mL			
5	2.590±.003	93±1	11.52±.01	0.109±.001	0.28±.01
5	2.590±.003	92±1	11.52±.03	0.109± 0	0.28±.01
25	2.505±.003	94±1	10.46±.01	0.126±.001	0.35±.01
25	2.505±.001	93±1	10.44±.04	0.126± 0	0.35±.01
35	2.417±.001	93±1	6.95±.04	0.195±.001	0.63±.01
35	2.418±.001	95±1	7.00±.04	0.197±.005	0.62±.01
50*	1.809±.001	16±1	1.76±.01	0.117±.001	
50*	1.902±.001	21±1	1.88±.02	0.158±.007	
50*	2.283±.006	56±1	2.66±.01	0.298±.005	0.62±.06
50*	1.808±.004	16±1	1.77±.02	0.125±.017	
50*	1.900±.004	22±1	1.91±.01	0.168±.001	
50*	2.280±.003	57±1	2.68±.01	0.301±.002	0.51±.06

* denotes split peaks

Table Xb

Chromatographic Results for MBC when [CH_3CN] in the Sample Solvent increases from 5 to 50% and Injected Mass of Analytes Increases with Increasing Injection Volume.

CH3CN CONC. %	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10 µL @ 5µg/mL			
5	3.323±.003	104±6	9.16±.11	0.163±.010	0.42±.03
5	3.326±.001	98±4	8.92±.19	0.156±.001	0.42±.01
25	3.329±.003	104±1	8.62±.09	0.169±.003	0.40±.02
25	3.332±.003	106±1	8.72±.01	0.170± 0	0.41± 0
35	3.317±.002	107±3	8.60±.08	0.176±.005	0.41±.01
35	3.320±.003	105±2	8.47±.16	0.178±.002	0.40±.01
50	3.316±.004	101±3	8.15±.06	0.183±.007	0.42±.01
50	3.320±.002	104±8	8.46±.61	0.188±.010	0.41±.04
		50 µL @ 5µg/mL			
5	3.393±.002	122±5	10.84 ±.41	0.182±.013	0.39±.01
5	3.393±.001	125±5	10.44 ±.15	0.172±.003	0.36±.02
25	3.367±.001	117±0	8.86 ±.01	0.196±.001	0.38±.01
25	3.366±.003	117±1	8.87 ±.04	0.197±.002	0.38±.01
35	3.343±.002	120±2	8.20 ±.10	0.216±.007	0.39±.02
35	3.345±.002	120±5	8.10 ±.10	0.216±.006	0.41± 0
50	3.316±.002	117±1	7.14 ±.03	0.243±.015	0.45±.01
50	3.319±.004	116±1	7.12 ±.01	0.247±.002	0.45±.01
		100µL @ 5µg/mL			
5	3.458±.004	120 ± 1	10.45 ± .01	0.165±.001	0.34 ± .01
5	3.461±.003	120 ± 1	10.43 ± .04	0.167±.006	0.34 ± .01
25	3.419±.002	122 ± 5	8.77 ± .03	0.202±.006	0.37 ± .02
25	3.414±.002	121 ± 1	8.69 ± .01	0.206±.001	0.38 ± .01
35	3.378±.002	122 ± 2	7.47 ± .06	0.239±.009	0.45 ± .02
35	3.373±.003	122 ± 1	7.42 ± .02	0.249±.001	0.44 ± .01
50	3.323±.001	121 ± 2	5.51 ± .01	0.342±.009	0.54 ± .01
50	3.374±.009	121 ± 2	5.54 ± .04	0.343±.002	0.59 ± .07
		200µL @ 5µg/mL			
5	3.571±.004	120 ± 1	10.11 ± .04	0.167±.001	0.31± 0
5	3.572±.002	120 ± 1	10.09 ± .03	0.167±.001	0.31±.01
25	3.498±.002	120 ± 1	7.94 ± .01	0.227±.001	0.42± 0
25	3.497±.002	120 ± 1	7.93 ± .01	0.227±.001	0.42±.01
35	3.432±.003	121 ± 1	5.90 ± .03	0.322±.002	0.53±.01
35	3.433±.002	123 ± 4	5.98 ± .08	0.321±.005	0.53±.01
50	3.329±.003	122 ± 2	3.54 ± .03	0.530±.040	0.68±.07
50	3.322±.005	123 ± 1	3.56 ± .01	0.569± 0	0.63±.06

Figure 13: Chromatograms of STB (1) and MBC (2) for Increasing Mass Study Showing the Effects of the Increasing Acetonitrile Concentration in the Sample Solvent. 1) 5% CH_3CN; 2) 25% CH_3CN; 3) 35% CH_3CN; 4) 50% CH_3CN.

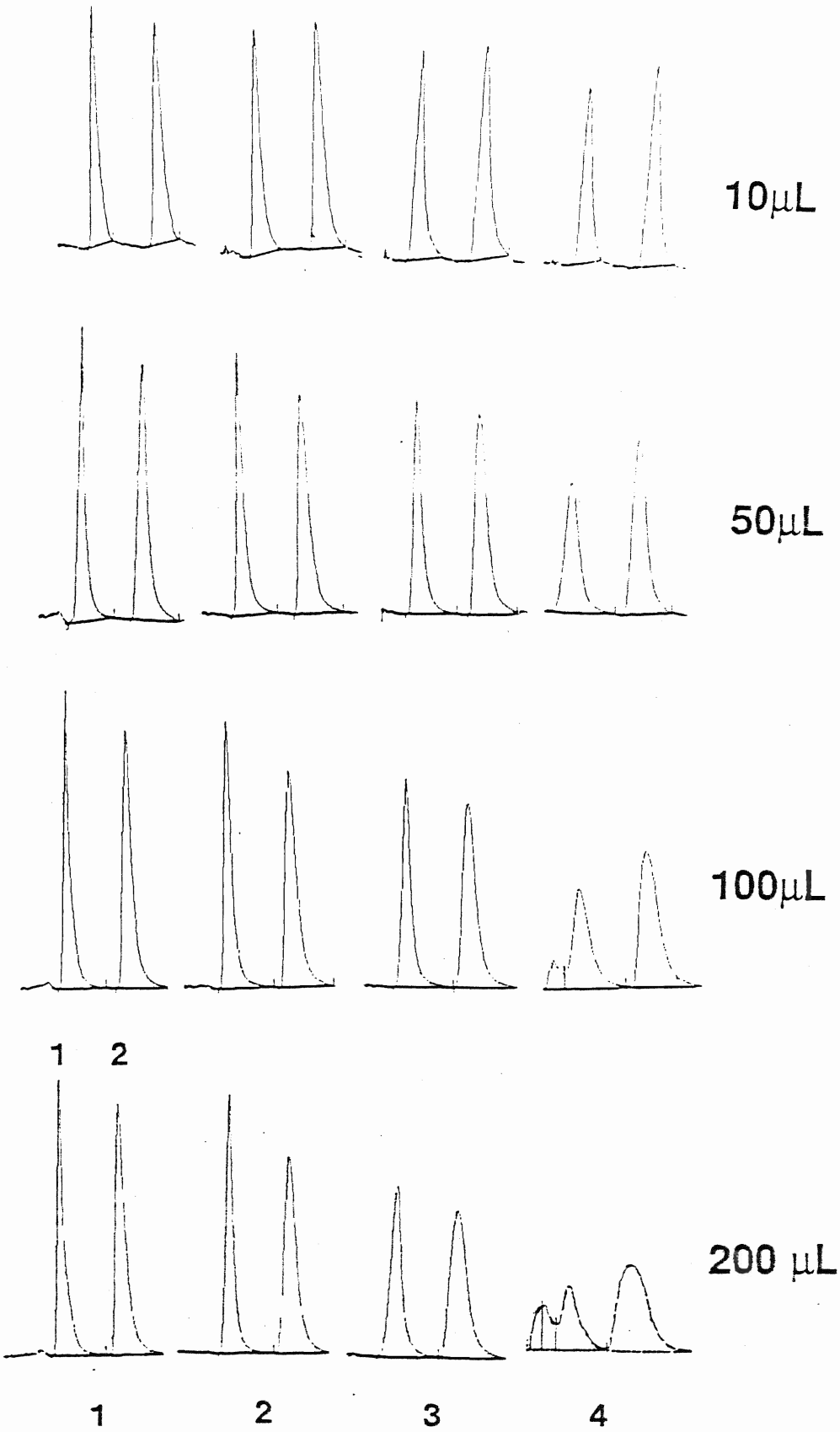


Figure 14: Plot of Retention Time vs. Acetonitrile Concentration in the Sample Solvent for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

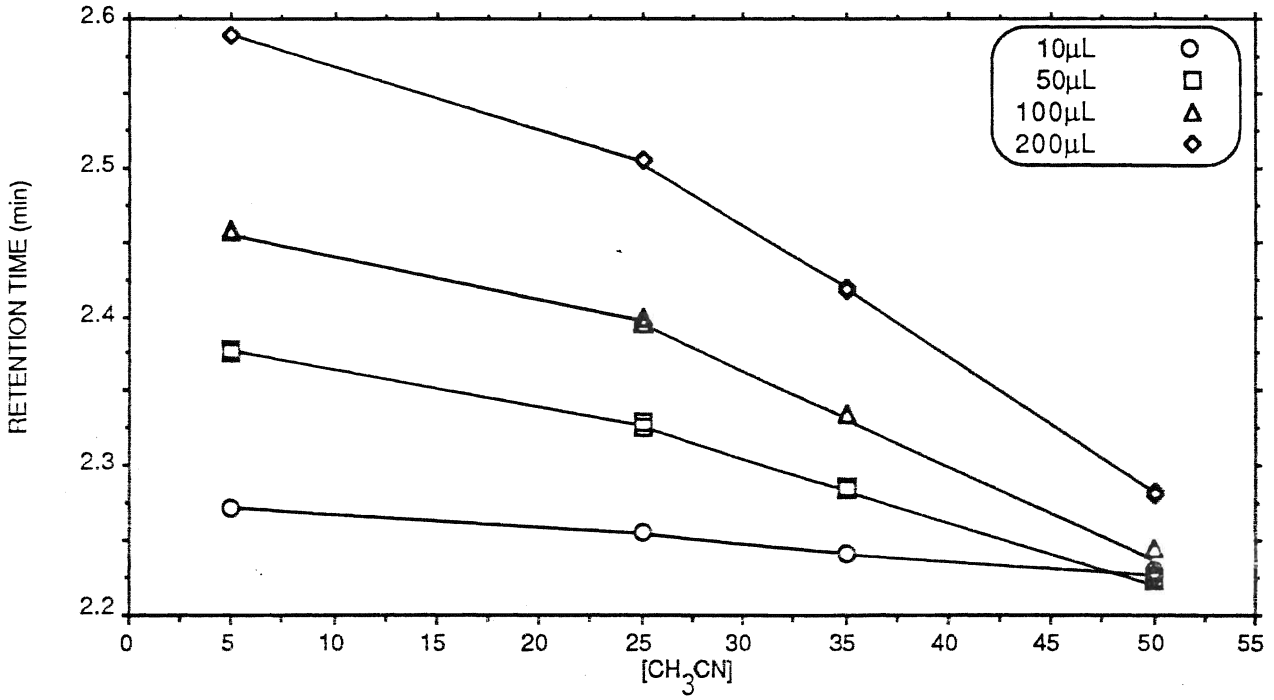


Figure 15: Plot of Retention Time vs. Acetonitrile Concentration in the Sample Solvent for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

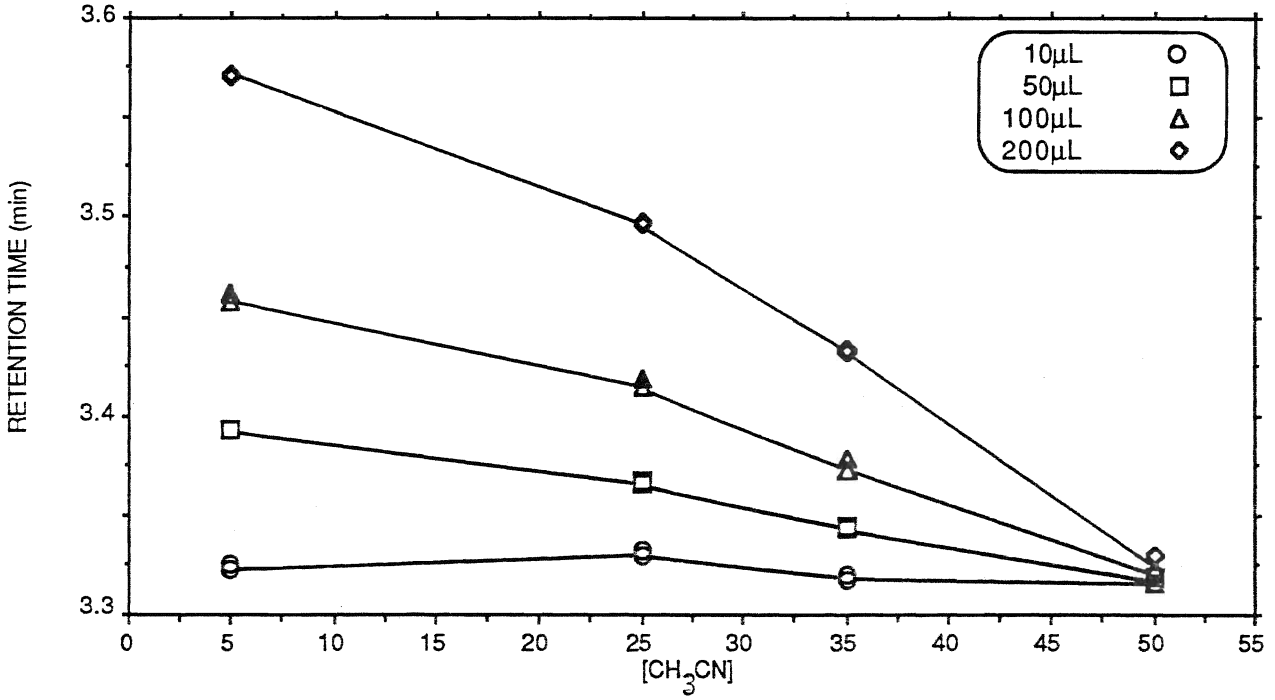


Figure 16: Plot of Peak Height vs. Acetonitrile Concentration in the Sample Solvent for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

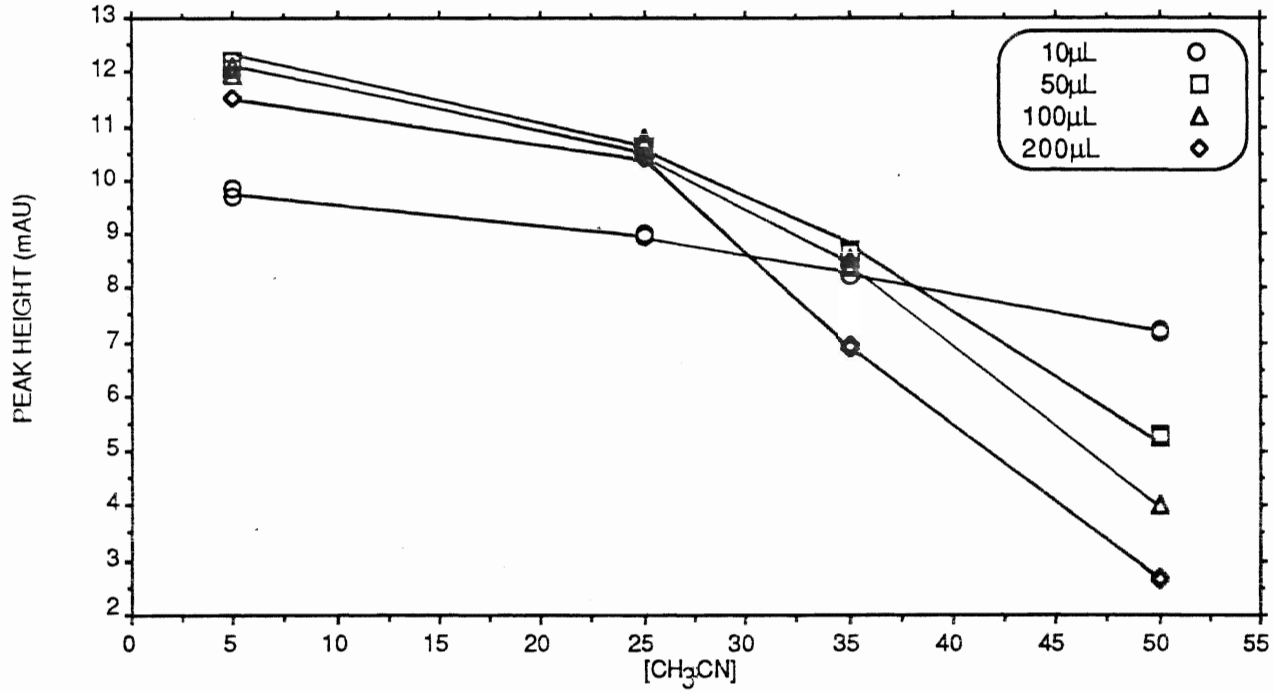


Figure 17: Plot of Peak Height vs. Acetonitrile Concentration in the Sample Solvent for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

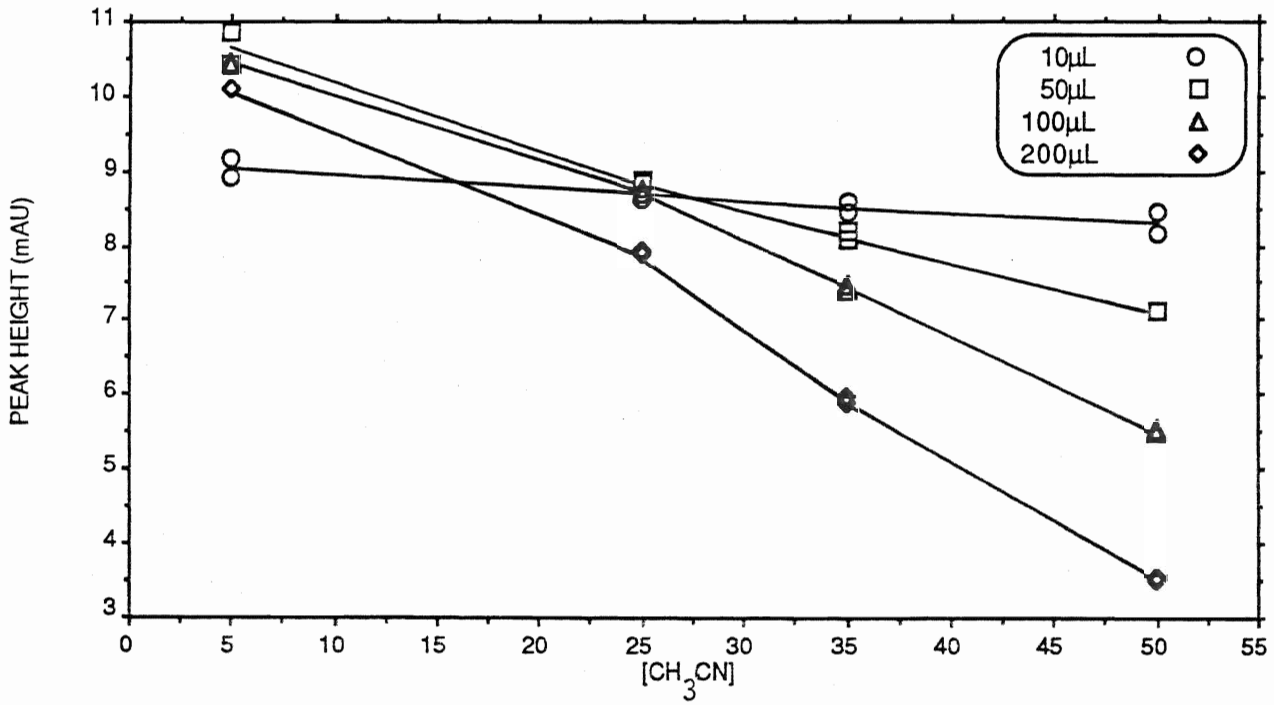


Figure 18: Plot of Peak Width vs. Acetonitrile Concentration in the Sample Solvent for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

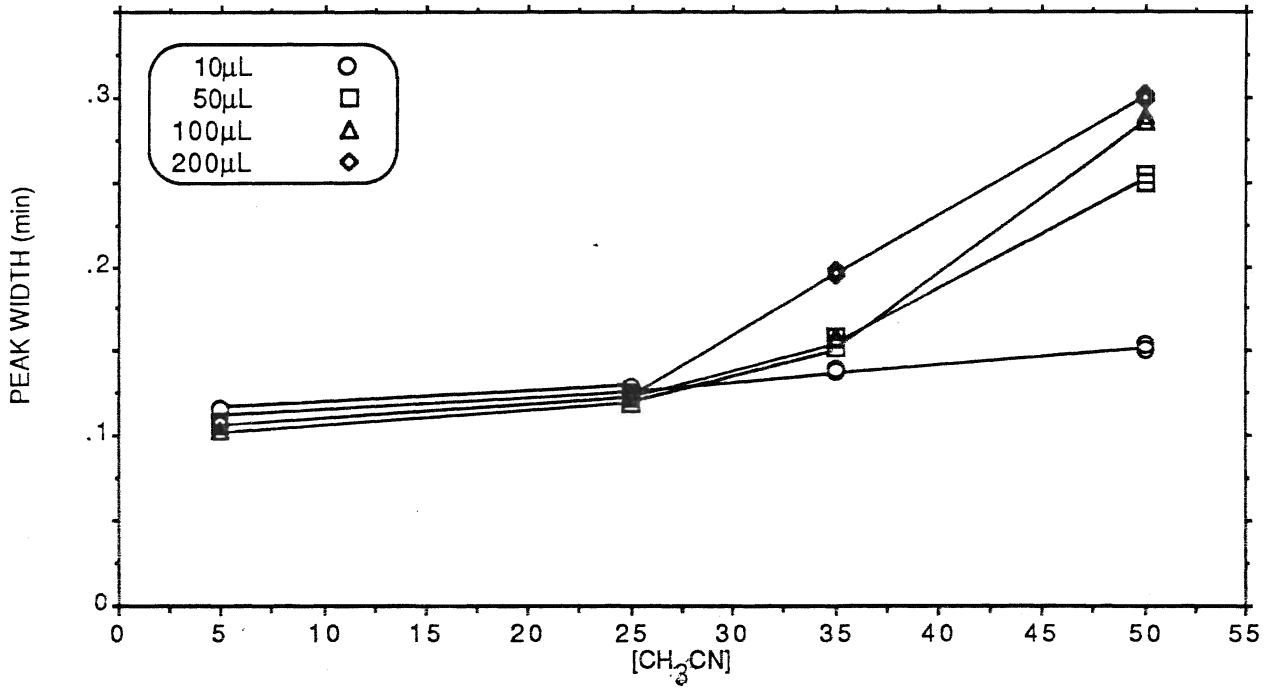
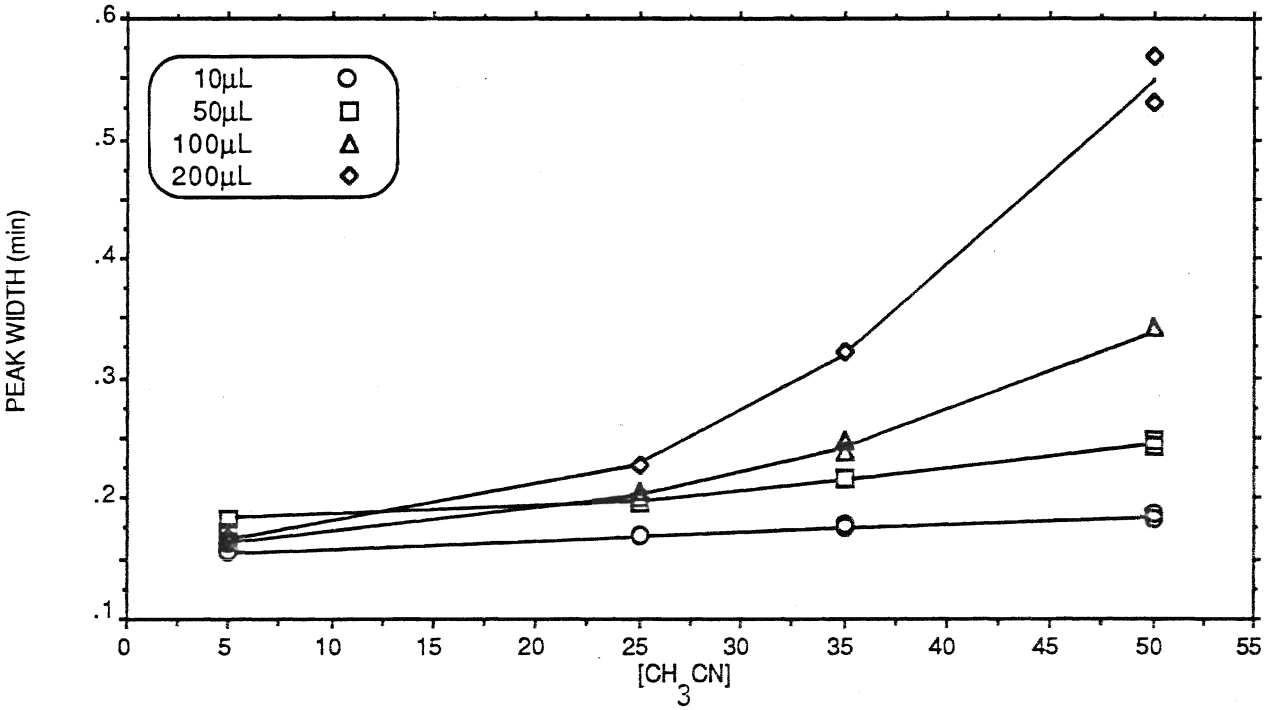


Figure 19: Plot of Peak Width vs. Acetonitrile Concentration in the Sample Solvent for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.



height results were devided by the factor of increase in the injection volumes. The resulting chromatographic plots are shown in Figure 13.

Retention Time The retention times of the two analytes were influenced in an almost identical way to the constant mass study. The decreases of 1.9, 6.4, 9.0 and 11.8% were observed for STB and decreases of 0.2, 2.2, 3.9, and 6.8% in retention times were observed for MBC for 10, 50, 100, and 200 µL injections. The decrease was more gradual in the range from 5 to 25% and became much steeper from 25 to 50% acetonitrile, for both analytes studied, as can be seen in Figures 14 and 15. For 50% acetonitrile in the 200 µL study, STB was eluted in two well defined peaks. The confirmation that the two peaks were due to STB was made by the UV scan of each peak.

Peak Height The peak heights of the two analytes were influenced to the same extent as for the constant mass study as can be observed in Figures 16 and 17. The decreases in the peak heights of STB were 26.3, 56.5, 66.5 and 76.8% while for MBC decreases were 8.1, 33.0, 47.1, and 64.9%.

Peak Width The peak widths increased with the increase in the acetonitrile content (Table Xa). At 5 and 25% acetonitrile, peak widths for STB were very similar at all four injection volumes, but sharply increased at higher acetonitrile concentrations (Figure 18). The increasing trend in the peak widths was also observed for MBC as can be seen in Figure 19. The influence of injection volume on the peak width of MBC became more apparent at 25% acetonitrile at which point there

was essentially no influence for STB (Figures 18 and 19).

Peak Area The areas of STB peaks were constant from 5 to 35%, and decreased only at 50% acetonitrile. However, this was due primarily to the presence of the split peaks (Table Xa). The areas of the MBC peaks were not affected at any acetonitrile concentrations (Table Xb).

Peak Symmetry An increase in the peak symmetry was observed at increasing acetonitrile concentrations. The increases were somewhat smaller for MBC than for STB and became more pronounced with an increase in the injection volume (Table Xa and Xb).

B. Effects of Methanol Concentration in the Sample Solution on the Chromatographic Peak Profile

1.Analysis on PE-HPLC

There was no apparent influence of methanol increase on the retention times of analytes at 50 µL injection volume and 0.06M buffer concentration. The influence on the peak heights, however, was quite pronounced and was different for the two analytes (Figure 27). The peak heights for STB increased by 15.1% as the percentage of methanol increased from 5 to 25%. In contrast to STB, peak heights for MBC decreased by 2.8% with the methanol increase. For both analytes, peak widths were not affected significantly enough to observe any changes

Table XI*

Chromatographic Results for STB and MBC when [CH_3OH] Increased from 5 to 25% at 0.06M Buffer Concentration

CH_3OH %	Retention Time (min) STB	MBC	Peak Height (cm) STB	MBC
5.0	3.50±.00	4.56±.02	10.16±.08	8.85±.09
10.0	3.56±.02	4.54±.02	10.08±.08	8.86±.12
12.5	3.56±.01	4.54±.02	10.25±.05	8.81±.13
15.0	3.56±.02	4.56±.02	11.23±.03	8.98±.02
20.0	3.56±.04	4.54±.04	11.28±.03	8.77±.06
25.0	3.44±.02	4.52±.04	11.69±.06	8.60±.04

*—done on PE-HPLC at 50µL injection volume

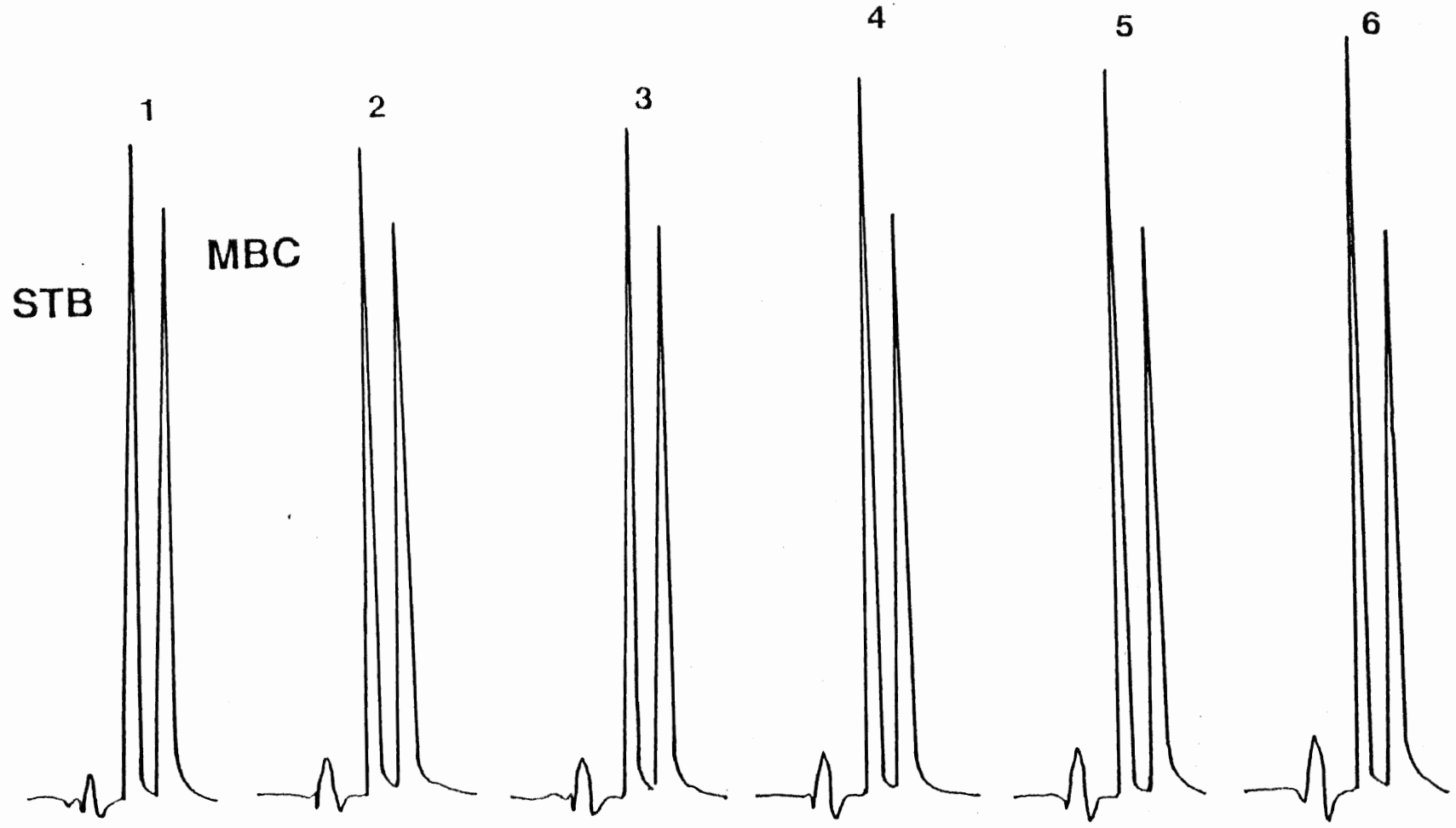


Figure 20: Chromatograms of STB (1) and MBC (2) Showing the Effects of Increasing Methanol Concentration in the Sample Solvent, at 50µL Injection Volume and 0.06M Buffer Concentration. 1) 5% CH_3OH; 2) 10% CH_3OH; 3) 12.5% CH_3OH; 4) 15% CH_3OH; 5)20% CH_3OH; 6) 25% CH_3OH.

at increasing methanol concentrations. The above results are listed in Table XI.

2.Analysis on HP-HPLC

Two factors were different in the basic solution composition from the PE study. The buffer concentration was changed from 0.06M to 0.007M and the methanol concentration range was increased to 5-50% from 5-25%.

1) At Constant Mass of Analyte Injected

Retention Time A retention time increases of 0.6, 2.1, 1.1, and 0.4% were observed for STB at the injection volumes of 10, 50, 100, and 200 µL respectively (Table XIIa). The increasing trend was less pronounced for the smallest and the largest injection volumes as can be seen in Figure 21. For MBC, retention times stayed constant as methanol increased from 5 to 50% for 10 µL injections, and very gradually decreased by 0.9, 1.3, and 2.4% for 50, 100, and 200 µL injections, respectively (Table XIIb and Figure 22). An interesting point is that STB retention time increased with an increasing amount of methanol in the sample solvent and MBC retention time decreased. This resulted in an improved peak resolution.

Peak Height STB peak heights increased by 2.9, 26.7, and 15.4% for 10, 50, and 100 µL injections as methanol increased from 5 to 50%. For 200 µL injections, peak heights increased by 15.1% from 5 to 35% methanol and then decreased by 25.6%

for a methanol increase from 35 to 50% (Table XIIa and Figure 23). It is important to note, that at the lower injection volumes the influence of methanol is opposite to that of acetonitrile, but that at sufficiently high injection volumes (200 µL) and high methanol content (50%), a decrease in the peak height was observed as were the cases with all the acetonitrile experiments.

MBC peak heights decreased with an increase in the methanol concentration. For all four injection volumes, the rate of decrease was slower from 5 to 25% methanol and more rapid from 35 to 50% as can be observed in Figure 24. The decreases of 5.1, 5.5, 15.3, and 30.2% were observed for 10, 50, 100, and 200 µL injections (Table XIIb).

Peak Width A decrease in the STB peak widths was observed as the methanol concentration increased. This trend was interrupted only for 200 µL injection at 50% methanol as can be observed in Figure 25. For MBC, peak widths increased with an increase in the methanol concentration. The increases became larger at higher injection volumes as shown in Figure 26. An interesting point is that at 5% methanol in the sample solvent, peak widths decreased with an increase in the injection volume, while at 50% methanol, peak widths were the same for 10 and 50 µL injections, but increased for the 100 and 200 µL injections (Table XIIb).

Peak Area The peak areas of the two analytes at all four injection volumes remained constant as methanol concentration in the sample solvent increased as shown in Tables XIIa and

Table XIIa

Chromatographic Results for STB when [MeOH] increases from 5 to 50% and Injected Mass of Analytes Stays Constant at Increasing Injection Volumes.

%MeOH	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
			10µL @ 5µg/mL		
5	2.452±.003	83±1	7.08±.01	0.164±.002	0.53±.01
5	2.453±.004	83±1	7.04±.03	0.165±.002	0.54±.01
25	2.459±.005	86±3	7.27±.10	0.169±.009	0.56±.01
25	2.460±.002	84±1	7.23±.06	0.171± 0	0.57±.01
35	2.468±.002	84±1	7.15±.08	0.172±.001	0.56±.01
50	2.469±.001	87±1	7.39±.01	0.169±.004	0.58±.01
50	2.468±.004	84±4	7.14±.12	0.173±.004	0.60±.01
			50 µL @ 1µg/mL		
5	2.555±.001	98±2	7.31±.01	0.171±.001	0.41±.01
5	2.550±.010	91±4	7.20±.13	0.168±.002	0.39±.01
25	2.608±.010	85±5	7.98±.06	0.148±.003	1.11±.01
25	2.607±.003	92±1	7.92±.01	0.153±.001	1.07±.01
35	2.592±.001	91±1	8.60±.01	0.145±.002	0.91±.01
35	2.599±.003	92±1	8.56±.02	0.146±.001	0.91±.01
50	2.608±.002	91±1	9.23±.08	0.135±.001	0.99±.03
50	2.605±.007	92±1	9.15±.02	0.136±.001	1.01±.02
			100 µL @ .5µg/mL		
5	2.672±.007	92±1	7.75±.04	0.160±.007	0.86±.03
5	2.669±.001	92±1	7.72±.01	0.161±.002	0.88±.03
25	2.688±.001	95±3	8.49±.01	0.149±.003	1.13±.03
25	2.686±.004	94±1	8.28±.08	0.146±.008	1.21±.01
35	2.686±.001	95±1	8.51±.10	0.147± 0	1.10±.06
35	2.686±.004	92±1	8.26±.01	0.149±.001	1.17±.03
50	2.698±.005	91±1	8.97±.07	0.135± 0	1.29±.02
50	2.698± 0	90±1	8.88±.01	0.132±.003	1.32±.01
			200µL @ .25µg/mL		
5	2.799±.009	109 ±1	10.81±.06	0.154±.004	0.65±.01
5	2.796±.001	107 ±1	10.76±.01	0.153±.003	0.66±.01
25	2.798±.004	114 ±2	11.88±.03	0.130±.002	0.82±.01
25	2.802±.002	112 ±1	11.74±.09	0.130± 0	0.85±.01
35	2.798±.004	114 ±2	12.51±.03	0.124±.002	0.82±.01
35	2.799±.003	114 ±1	12.31±.06	0.126±.001	0.83±.01
50	2.811±.001	100 ±2	9.47±.14	0.147± 0	1.14±.03
50	2.806±.005	101 ±2	9.00±.63	0.148±.001	1.11±.01

Table XIIb

Chromatographic Results for MBC when [MeOH] in the Sample Solvent increases from 5 to 50% and Injected Mass of Analytes Stays Constant at Increasing Injection Volumes.

%MeOH	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
			10µL @ 5µg/mL		
5	3.322±.001	108±1	6.39±.04	0.234±.002	0.52 ±.02
5	3.322±.003	112±2	6.44±.01	0.238±.012	0.51 ±.01
25	3.320±.010	108±5	6.30±.13	0.239±.002	0.53 ±.01
25	3.323±.006	108±3	6.24±.02	0.245±.007	0.52 ±.01
35	3.332±.005	106±2	6.15±.05	0.246±.002	0.53 ±.01
50	3.322±.007	105±2	6.10±.07	0.236±.006	0.53 ±.01
50	3.319±.004	109±3	6.08±.04	0.250±.011	0.52 ±.01
			50 µL @ 1µg/mL		
5	3.401±.002	114±2	7.38 ±.11	0.215±.007	0.55 ±.01
5	3.401±.012	117±5	7.57 ±.09	0.216±.005	0.54 ±.02
25	3.359±.003	115±2	7.12 ±.01	0.226±.006	0.55 ±.01
25	3.373±.013	114±3	7.13 ±.04	0.228±.007	0.58 ±.02
35	3.374±.001	118±1	6.64 ±.07	0.241±.007	0.54 ±.01
35	3.371±.008	118±3	6.74 ±.23	0.238±.001	0.57 ±.01
50	3.370±.006	118±2	7.07 ±.03	0.235±.002	0.55 ±.01
50	3.370±.006	116±1	7.06 ±.07	0.234±.002	0.55 ±.02
			100 µL @ .5µg/mL		
5	3.466±.003	118±3	8.11 ±.06	0.210±.006	0.54 ±.01
5	3.465±.001	118±1	8.09 ±.02	0.209±.001	0.55 ±.01
25	3.447±.003	118±5	7.59 ±.09	0.222± 0	0.59 ±.03
25	3.451±.003	119±2	7.58 ±.01	0.223±.003	0.59 ±.01
35	3.433±.001	119±3	7.04 ±.11	0.232±.001	0.60 ±.01
35	3.430±.004	113±2	7.01 ±.10	0.227±.010	0.62 ±.01
50	3.420±.004	118±1	6.86 ±.02	0.244±.005	0.63 ±.03
50	3.423±.004	118±1	6.87 ±.01	0.241± 0	0.63 ±.02
			200µL @ .25µg/mL		
5	3.577±.004	141±3	10.66±.02	0.193±.001	0.51 ±.01
5	3.573±.003	143±2	10.60±.06	0.197±.003	0.50 ±.01
25	3.544±.005	145±1	9.99±.03	0.211±.005	0.60 ±.10
25	3.549±.002	147±2	9.99±.06	0.211±.004	0.57 ±.01
35	3.527±.003	145±2	9.07±.02	0.230±.005	0.61 ±.02
35	3.530±.002	145±3	9.00±.05	0.230±.007	0.61 ±.09
50	3.490±.002	139±6	7.40± 12	0.275±.005	0.69 ±.02
50	3.486±.001	145±1	7.44±.03	0.277±.003	0.67 ±.01

Figure 21: Plot of Retention Time vs. Methanol Concentration in the Sample Solvent for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

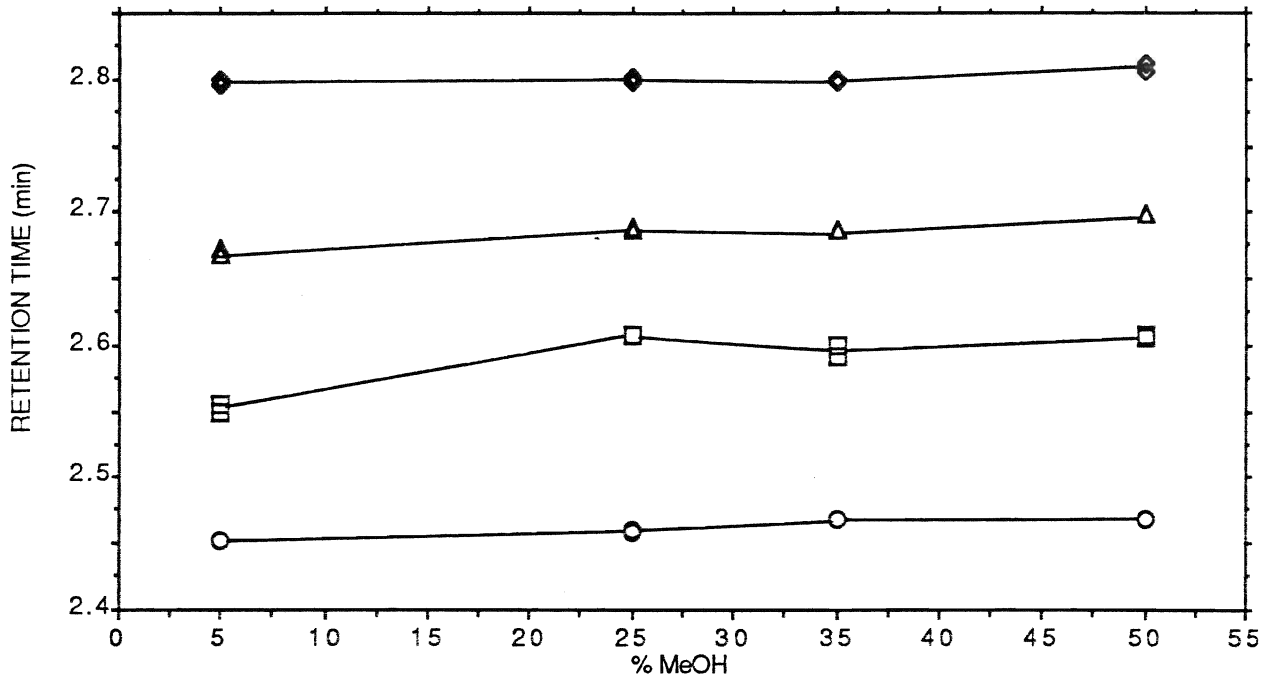


Figure 22: Plot of Retention Time vs. Methanol Concentration in the Sample Solvent for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

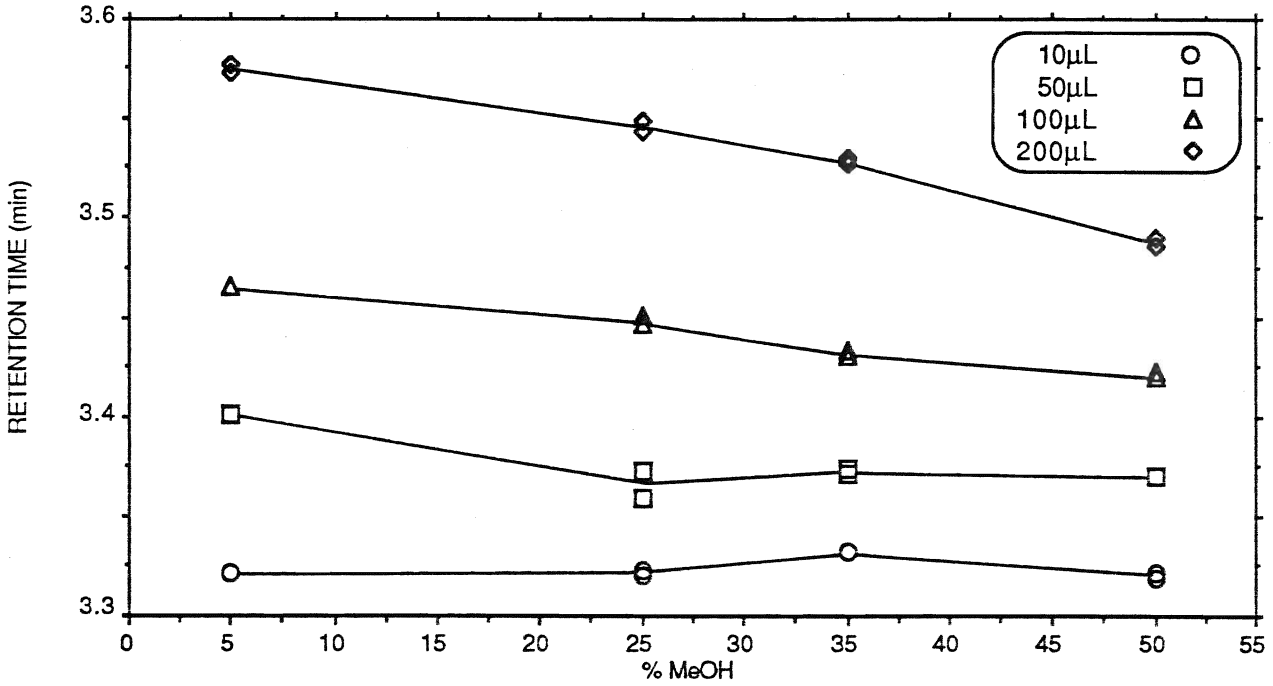


Figure 23: Plot of Peak Height vs. Methanol Concentration in the Sample Solvent for STB at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

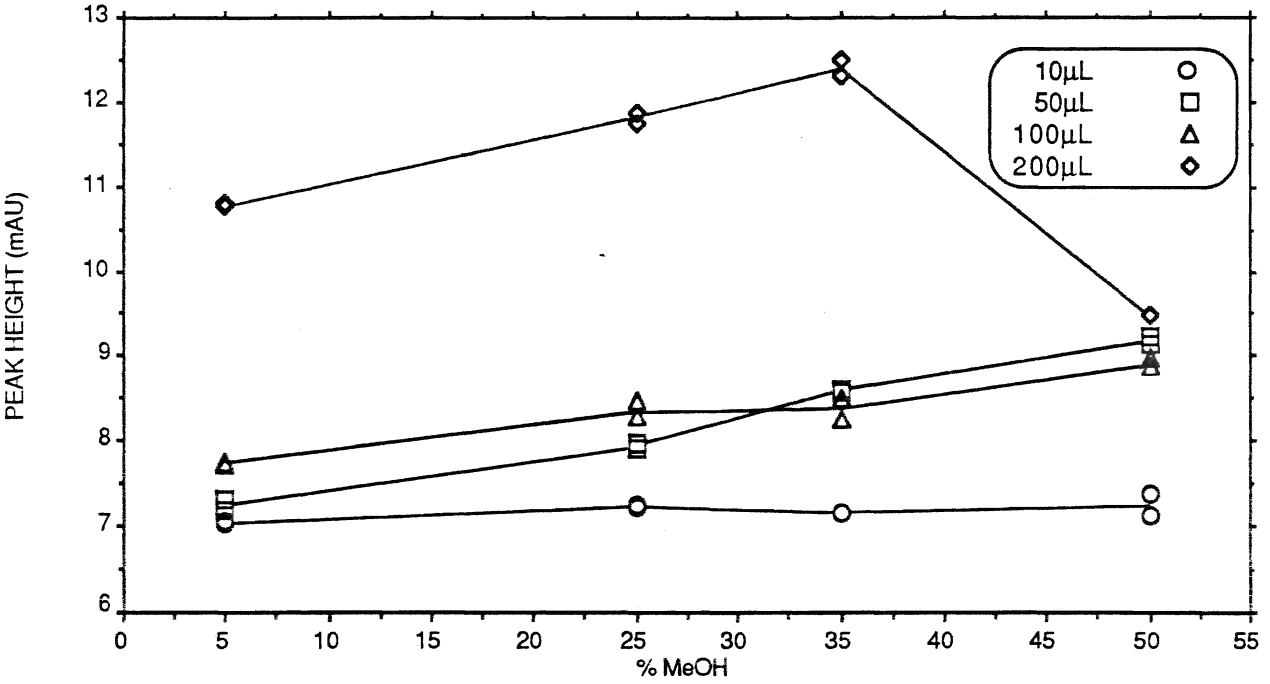


Figure 24: Plot of Peak Height vs. Methanol Concentration in the Sample Solvent for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

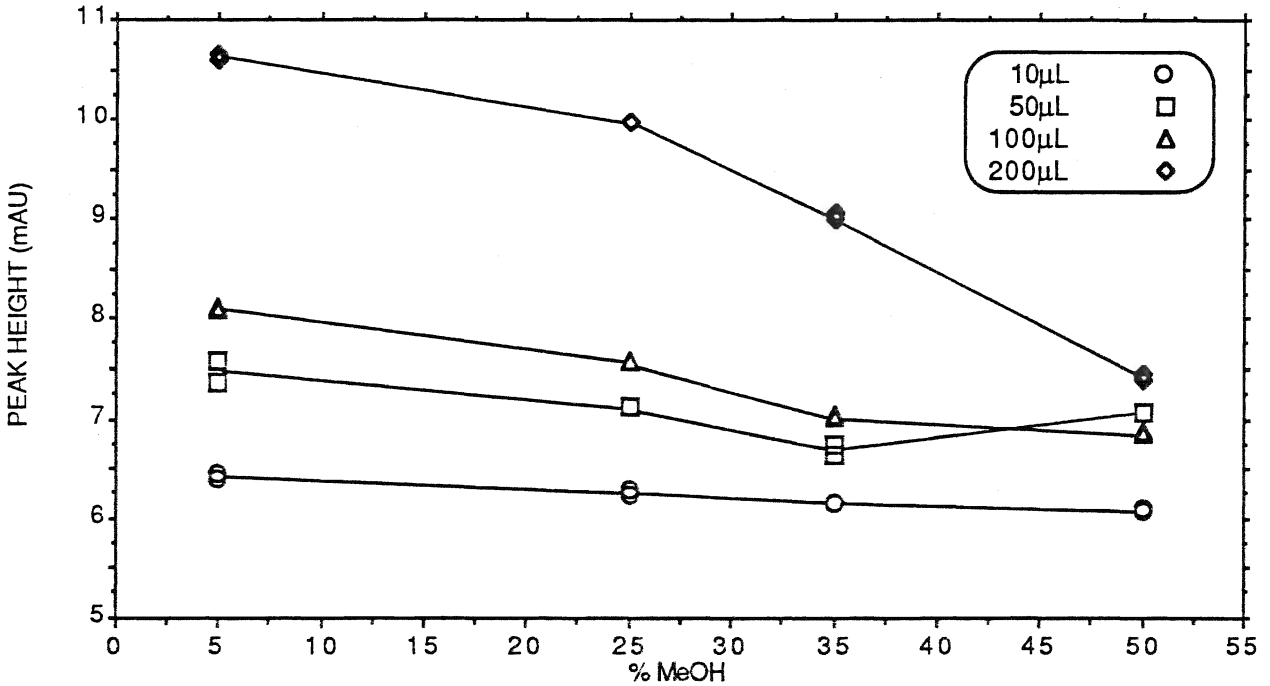


Figure 25: Plot of Peak Width vs. Methanol Concentration in the Sample Solvent for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

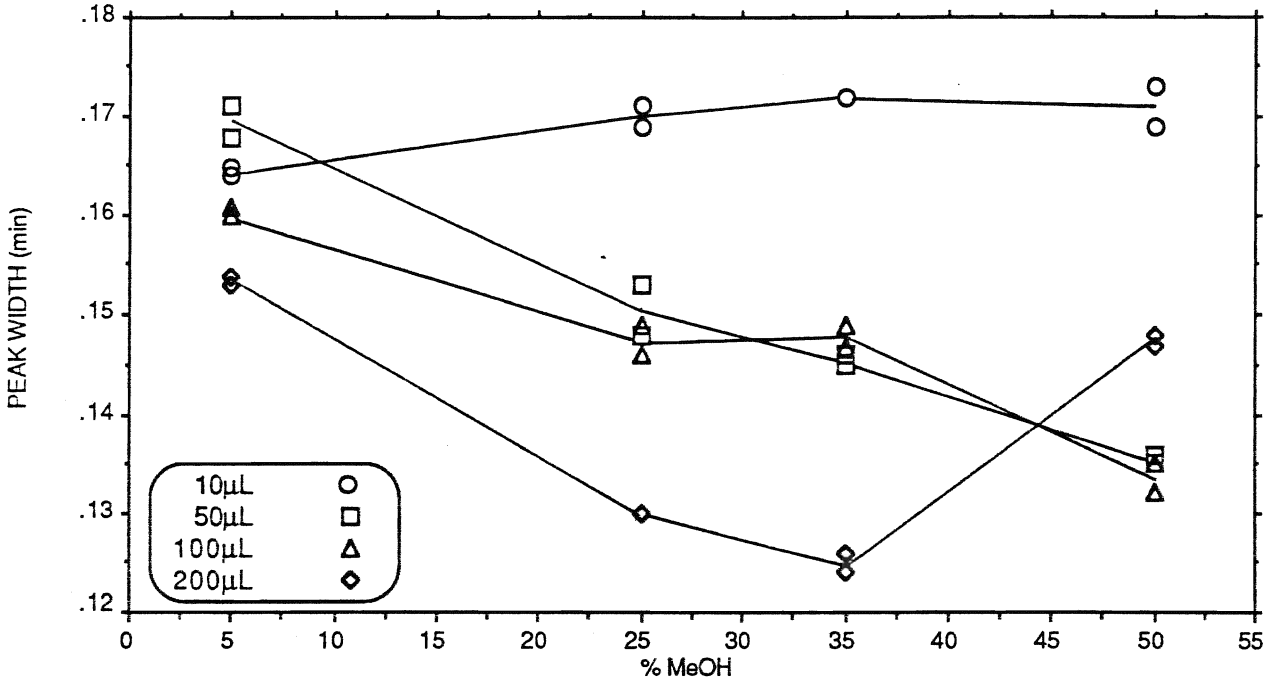


Figure 26: Plot of Peak Width vs. Methanol Concentration in the Sample Solvent for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

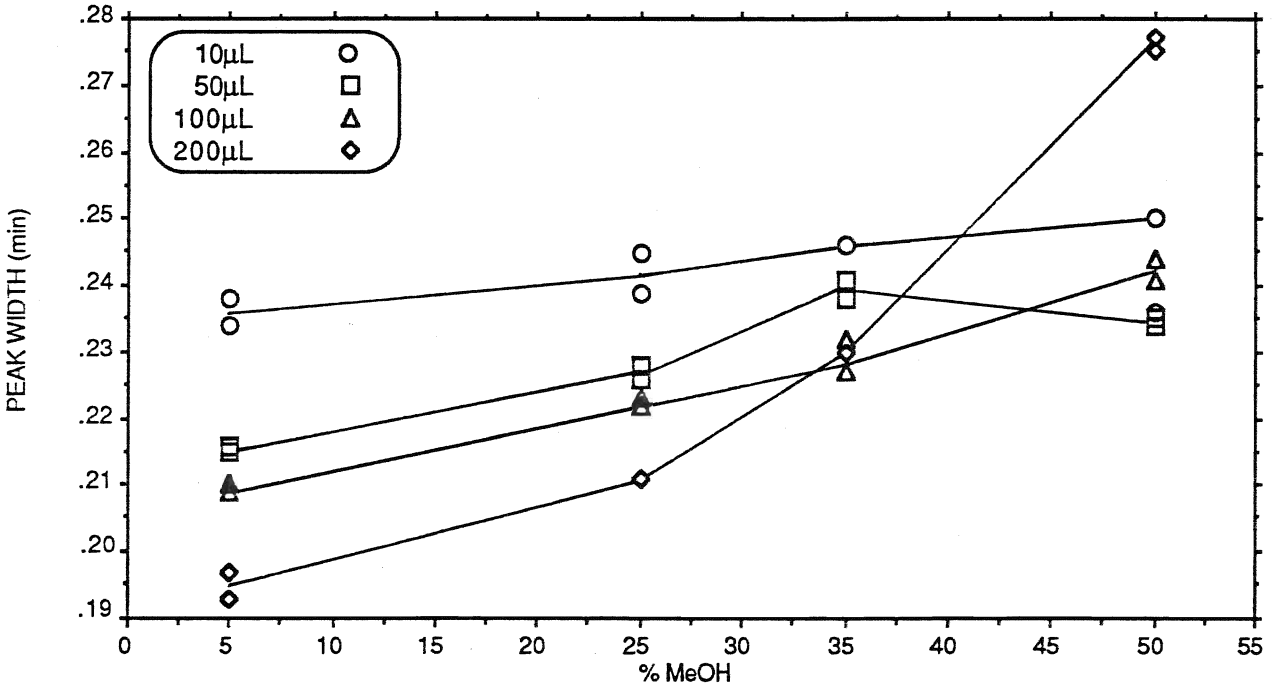
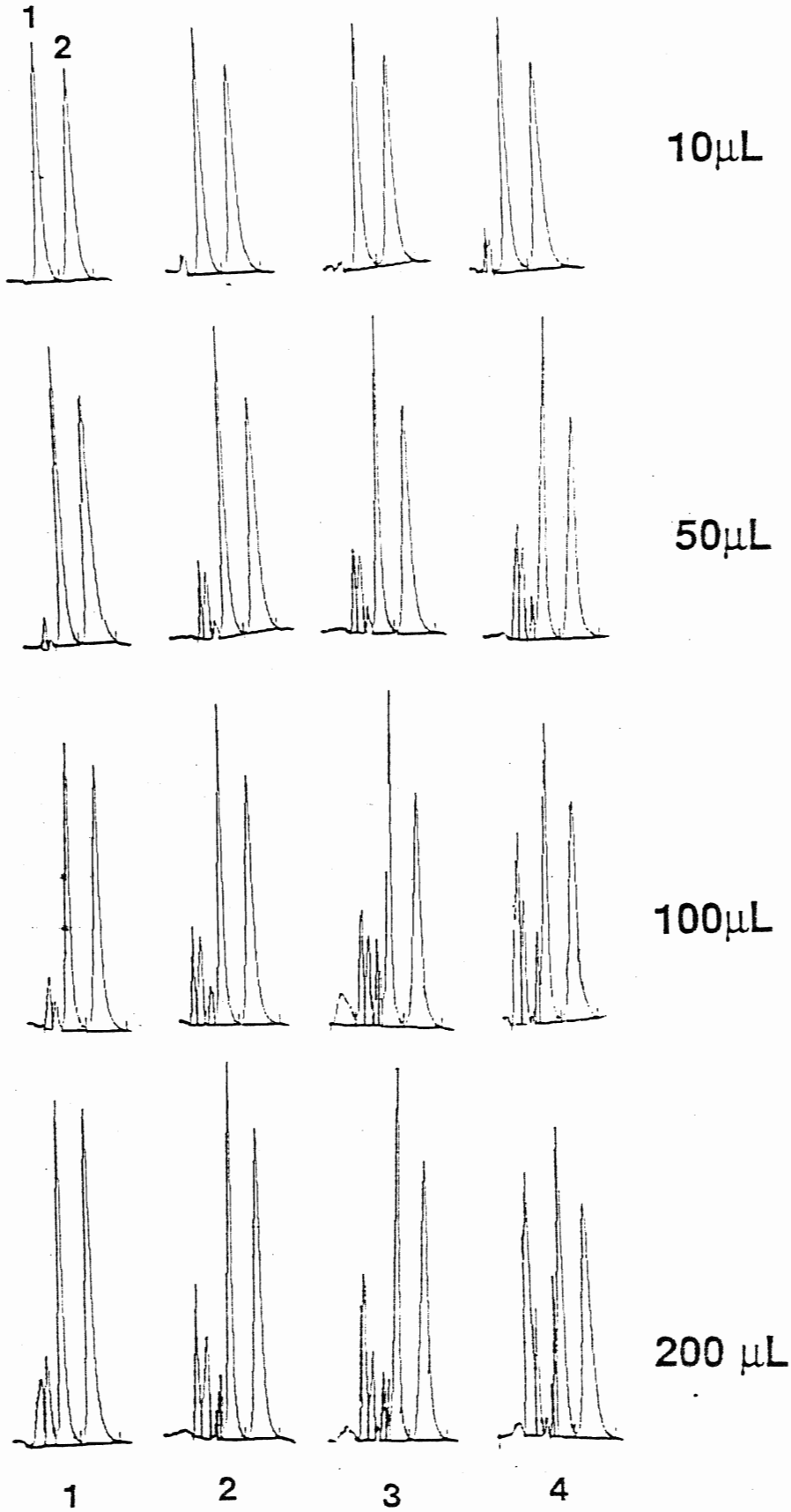


Figure 27: Chromatograms of STB (1) and MBC (2) for Constant Mass Study Showing the Effects of Increasing Methanol Concentration in the Sample Solvent on the Peak Profiles. 1) 5% CH_3OH; 2) 25% CH_3OH; 3) 35% CH_3OH; 4) 50% CH_3OH.



XIIb.

Peak Symmetry An increase in the peak symmetry was also observed with an increase in the methanol concentration. For STB, increases became larger with the increase in the injection volume. For MBC, peak symmetry stayed constant for the 10 and 50 µL injections and increased for the 100 and 200 µL injections as shown in Table XIIb.

2) At Increasing Mass of Analytes Injected

Retention Time The retention time of STB increased with an increase in methanol concentration. The increases of 0.7, 2.6, 4.5, and 6.3% were observed for 10, 50, 100, and 200 µL injections (Table XIIIa). While for constant mass study, increases became smaller at increasing volumes (Figure 21), at increasing mass, retention time increases became larger at higher injection volumes as shown in Figure 28. Another difference from the constant mass study was the occurence of split peaks. The split peaks were observed for the 100 µL injection at 25% methanol and for 200 µL injections at methanol concentrations of 25, 35, and 50%.

For MBC, the retention time for a 10 µL injection remained constant as methanol concentration increased. For 50, 100, and 200 µL injections, retention time decreased by 0.6, 2.5, and 3.0% with an increase in the methanol concentration as can be observed from Figure 29.

Peak Height A small increase in the STB peak height of 2.3% was observed for the 10 µL injection, and a much larger

Table XIIIa

Chromatographic Results for STB when [MeOH] in the Sample Solvent increases from 5 to 50% and Injected Mass of Analytes Increases with Increasing Injection Volume.

%MeOH	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
			10µL @ 5µg/mL		
5	2.452±.003	83±1	7.08±.01	0.164±.002	0.53±.01
5	2.453±.004	83±1	7.04±.03	0.165±.002	0.54±.01
25	2.459±.005	86±3	7.27±.10	0.169±.009	0.56±.01
25	2.461±.002	84±1	7.23±.06	0.171±0	0.57±.01
35	2.468±.002	84±1	7.15±.08	0.172±.001	0.56±.01
50	2.469±.001	87±1	7.39±.01	0.169±.009	0.58±.01
50	2.468±.004	84±4	7.14±.12	0.173±.004	0.61±.01
			50 µL @ 5µg/mL		
5	2.553	90	6.59	0.184	0.41
5	2.554	89	6.48	0.184	0.41
25	2.611	92	7.81	0.155	1.17
25	2.617	92	7.79	0.155	1.19
35	2.601	91	7.92	0.158	0.93
50	2.611	89	8.26	0.146	1.16
50	2.629	90	8.14	0.146	1.18
			100 µL @ 5µg/mL		
5	2.614	89	7.21	0.167	0.32
5	2.622	89	7.12	0.166	0.32
25*	2.631	45	5.35	0.134	1.71
25*	2.742	48	5.98	0.112	0.51
25*	2.636	46	5.36	0.138	1.74
25*	2.746	47	5.75	0.113	0.49
35	2.718	83	6.43	0.201	1.57
50	2.728	88	6.53	0.182	2.06
50	2.741	92	6.42	0.185	2.14
			200µL @ 5µg/mL		
5	2.743	90	7.03	0.175	0.34
5	2.743	88	6.89	0.175	0.35
25*	2.748	55	4.73	0.167	1.21
25*	2.813	33	3.63	0.123	0.43
25*	2.748	62	4.58	0.178	1.33
25*	2.817	26	3.41	0.124	0.41
35*	2.823	55	3.58	0.193	1.41
35*	2.916	38	4.19	0.128	0.65
50*	2.521	19	2.26	0.125	0.78
50*	2.914	72	4.14	0.231	2.27
50*	2.531	18	2.23	0.122	0.83
50*	2.918	73	4.16	0.233	2.31

Table XIIIb

Chromatographic Results for MBC when [MeOH] in the Sample Solvent increases from 5 to 50% and Injected Mass of Analytes Increases with Increasing Injection Volume.

%MeOH	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10µL @ 5µg/mL			
5	3.322±.001	108±1	6.39±.04	0.234±.002	0.52 ± .02
5	3.322±.003	112±2	6.44±.01	0.238±.012	0.51 ± .01
25	3.320±.010	108±5	6.30±.13	0.239±.002	0.53 ± .01
25	3.323±.006	108±3	6.24±.02	0.245±.007	0.52 ± .01
35	3.332±.005	106±2	6.15±.05	0.246±.002	0.53 ± .01
50	3.322±.007	105±2	6.10±.07	0.236±.006	0.53 ± .01
50	3.319±.004	109±3	6.08±.04	0.250±.011	0.52 ± .01
		50 µL @ 5µg/mL			
5	3.382	116	7.17	0.231	0.54
5	3.384	117	7.18	0.231	0.54
25	3.381	119	6.96	0.243	0.53
25	3.379	117	6.93	0.241	0.54
35	3.361	119	6.62	0.257	0.54
50	3.362	123	6.78	0.268	0.51
50	3.362	123	6.76	0.261	0.51
		100 µL @ 5µg/mL			
5	3.445	116	6.72	0.266	0.59
5	3.454	113	6.75	0.277	0.56
25	3.436	123	6.65	0.255	0.64
25	3.442	125	6.57	0.261	0.64
35	3.427	125	6.22	0.278	0.64
50	3.403	125	6.11	0.285	0.65
50	3.411	126	6.07	0.281	0.67
		200µL @ 5µg/mL			
5	3.617	122	6.31	0.302	0.41
5	3.616	124	6.31	0.306	0.41
25	3.556	125	5.33	0.365	0.88
25	3.552	124	5.13	0.378	0.89
35	3.554	126	4.86	0.345	1.01
50	3.503	125	4.49	0.392	0.94
50	3.511	125	4.91	0.398	0.95

Figure 28: Plot of Retention Time vs. Methanol Concentration in the Sample Solvent for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

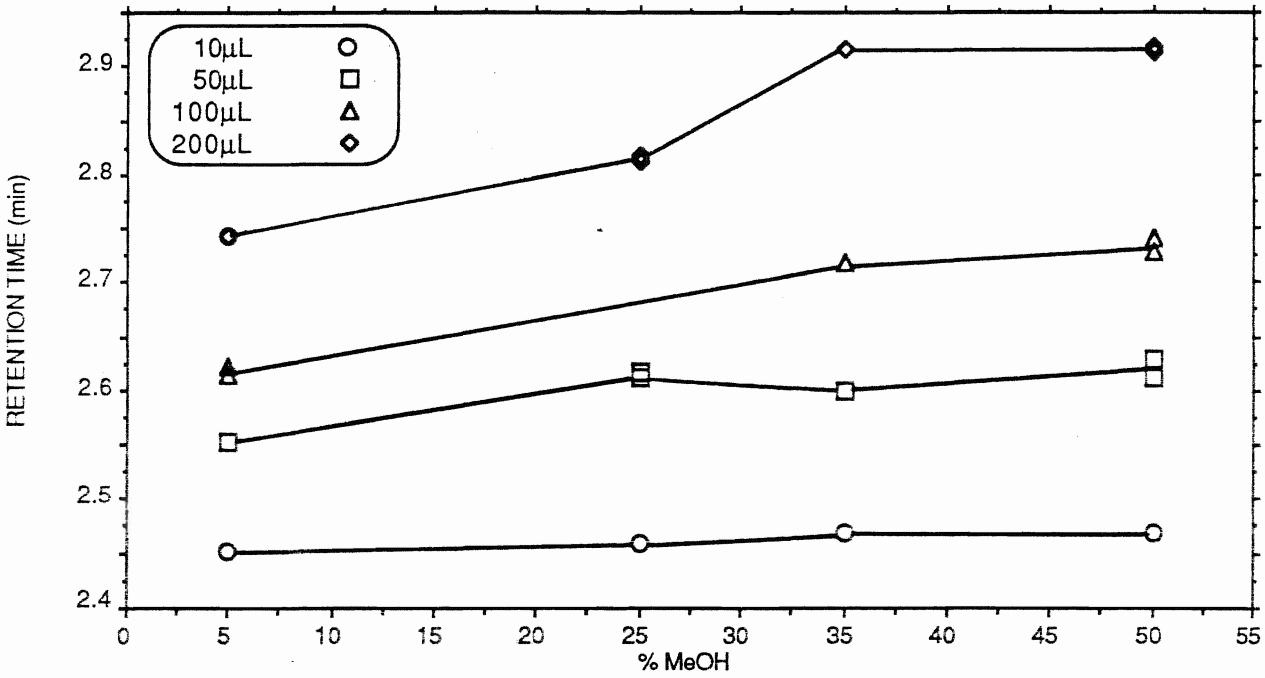


Figure 29: Plot of Retention Time vs. Methanol Concentration in the Sample Solvent for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

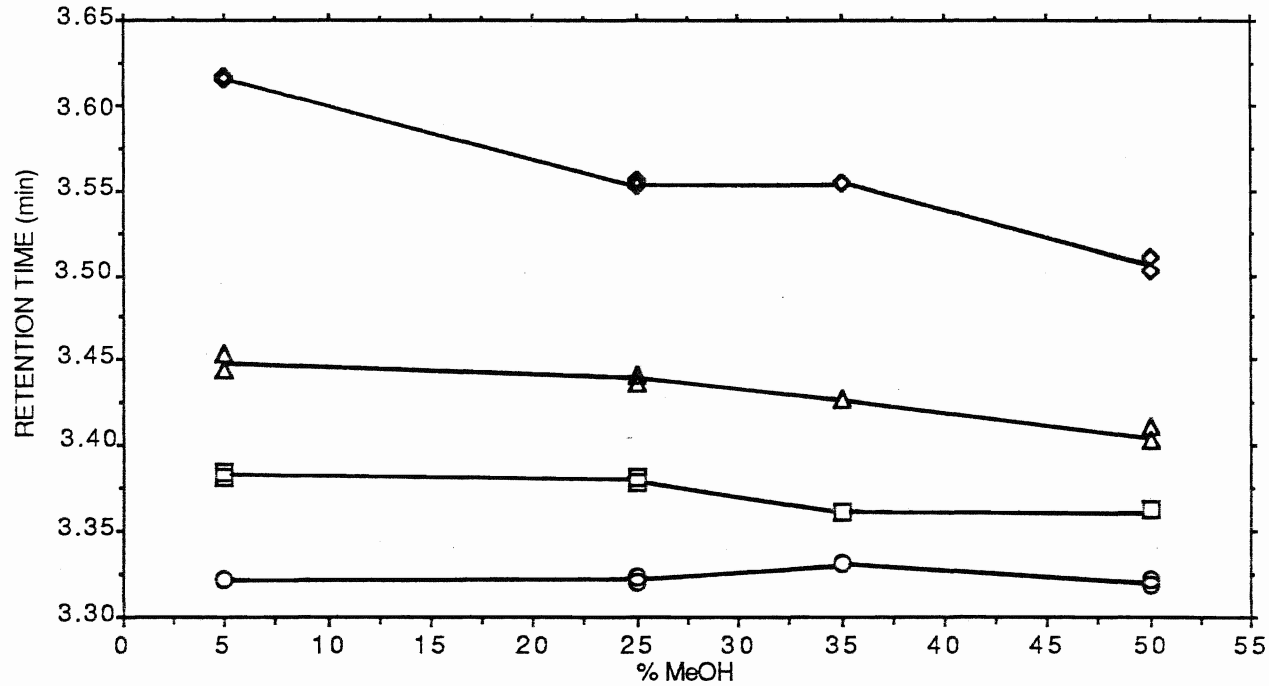


Figure 30: Plot of Peak Height vs. Methanol Concentration in the Sample Solvent for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

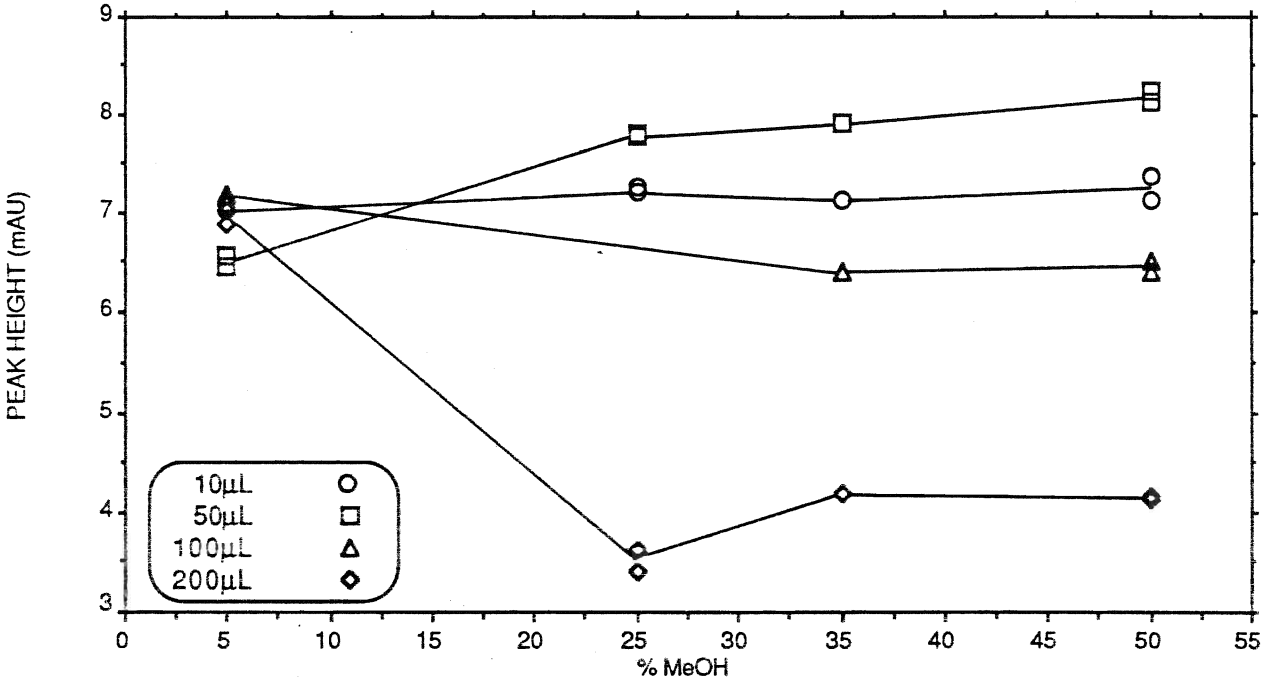


Figure 31: Plot of Peak Height vs. Methanol Concentration in the Sample Solvent for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

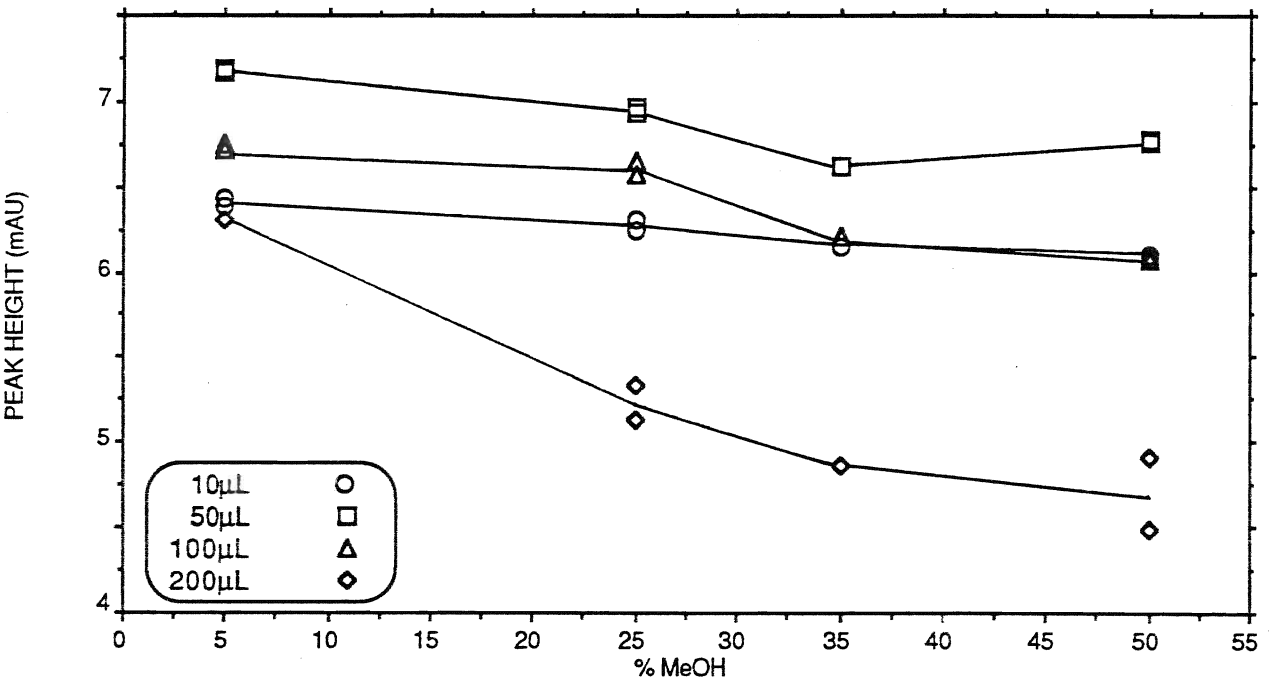


Figure 32: Plot of Peak Width vs. Methanol Concentration in the Sample Solvent for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

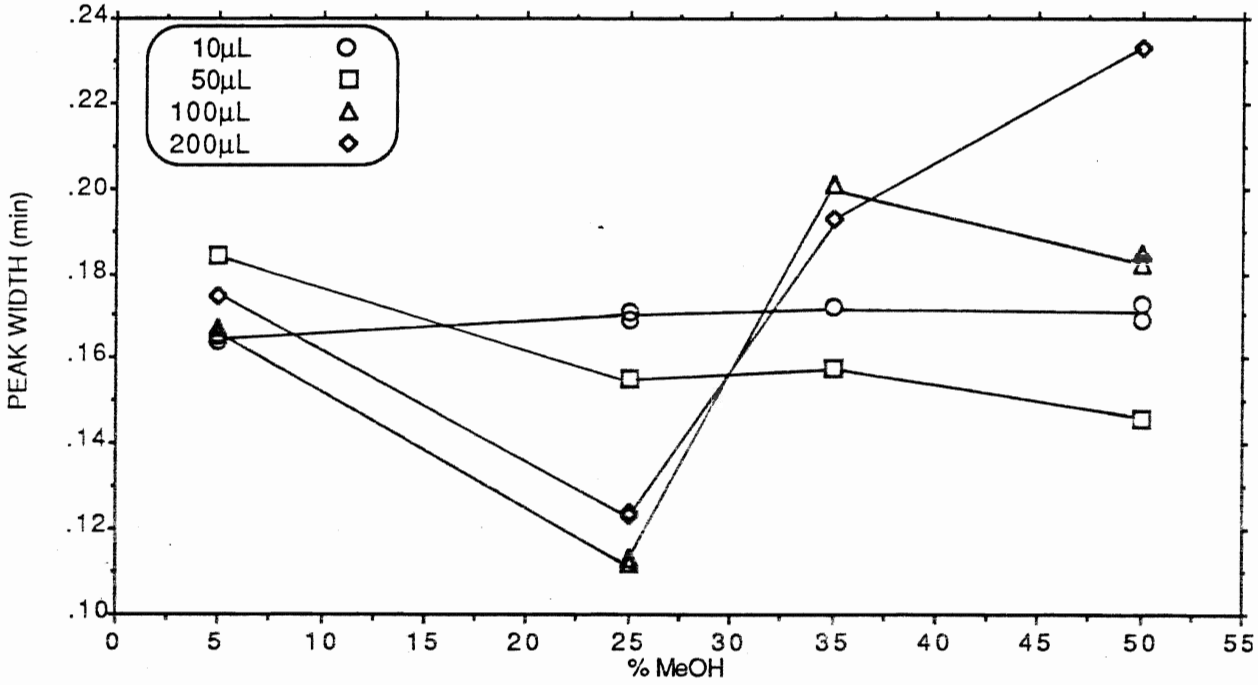
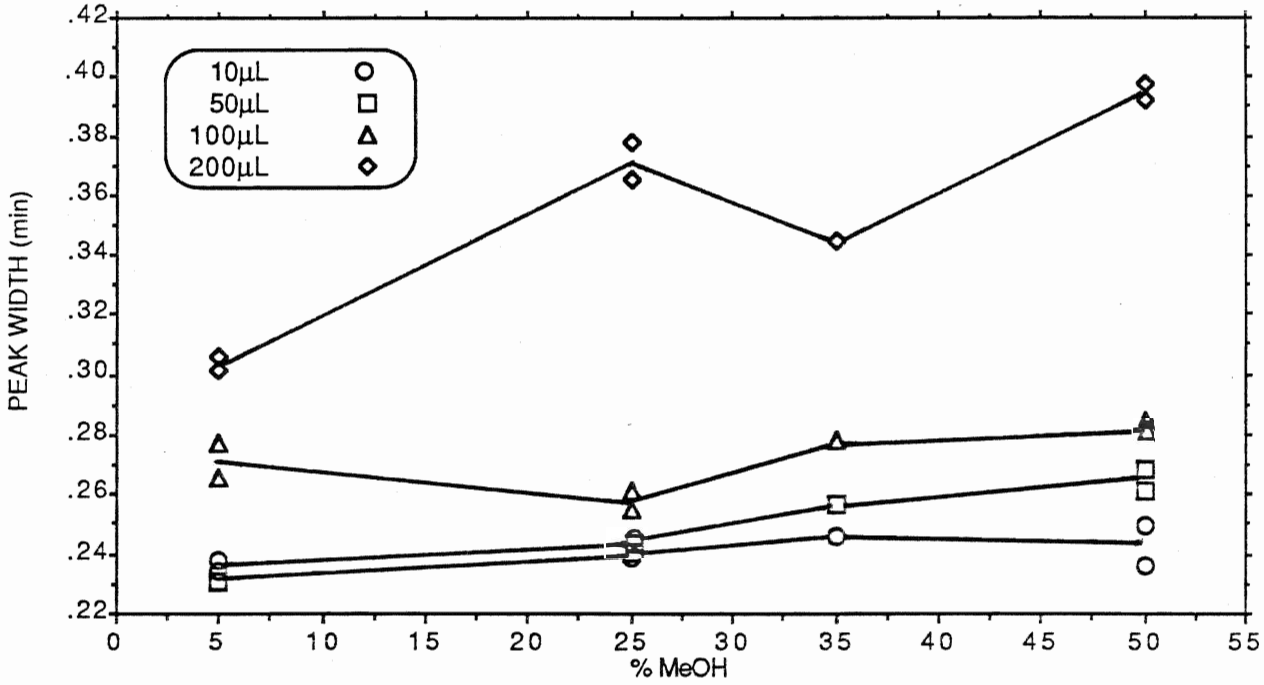


Figure 33: Plot of Peak Width vs. Methanol Concentration in the Sample Solvent for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.



increase of 25.4% was observed for the 50 µL injection. At 100 µL, peak height decreased by 9.5%, and at 200 µL injection, the decrease was even more pronounced and was accompanied by peak splitting. Results are listed in Table XIIIa and are graphically shown in Figure 30.

In the MBC study, peak heights decreased with methanol increase from 5 to 50% (Table XIIIb). The decreases of 5.3, 5.6, 9.5, and 25.5% were observed for 10, 50, 100, and 200 µL injections as shown in Figure 31. These decreases are similar to constant mass study for 10, and 50 µL injections, and are lower by around 5% for the 100 and 200 µL injections.

Peak Width There was no pronounced influence on the peak width of STB at 10 µL injection. For a 50 µL injection, peak width decreased with methanol increase, while for 100 and 200 µL injections, peak widths increased with an increase in the methanol concentration as shown in Figure 32. For MBC, an increasing trend in the peak width was observed, similar to that of constant mass study as shown in Figure 33.

Peak Area The peak area of STB was not affected by the methanol increase in the sample solvent for 10 and 50 µL injections, but at 100 and 200 µL injections, split peaks were observed. The areas of the two peaks, however, always equaled the area observed for the single peak, as can be seen in Table XIIIa. Peak area was not affected by the increase in the methanol concentration for any of the injection volumes studied for MBC (Table XIIIb).

Peak Symmetry For STB, peak symmetry increased at higher

methanol concentrations. The increases became larger as the injection volume increased (Table XIIIa). An increase in the peak symmetry for MBC was observed only at 100 and 200 µL injections.

C. Effect of the pH of the Sample Solution on the Chromatographic Peak Profile

1.Analysis on PE-HPLC

For this study the pH of the sample solutions was varied from 4.42 to 9.10. The injection volume for the analysis was 50 µL. The complete results are listed in Table XIV.

Retention Time A significant decrease in the STB retention time of 21.1% was observed as pH of the solution increased from 4.42 to 9.10 (Table IVX). At the pH of 4.42 the retention time of STB became so close to that of MBC that there was no resolution between the two peaks. The retention time of MBC was not affected by an increase in the pH of the sample solution.

Peak Height An increase in the pH of the sample solution resulted in the peak height increase of 80.9% for STB. For MBC however, peak height was not affected, except at pH 9.10, where a small decrease was observed (Table XIV).

Table XIV*

Influence of pH in the Sample Solvent on the Peak Profile in RP-HPLC

pH of Buffer	RETENTION TIME (min) for STB	RETENTION TIME (min) for MBC	PEAK HEIGHT (cm) for STB	PEAK HEIGHT (cm) for MBC
4.42	4.03±.02	4.03±.02	* *	* *
5.95	3.78±.03	4.03±.02	5.47±.03	12.05±.05
6.50	3.38±.03	4.04±.04	7.72±.07	10.55±.05
6.74	3.32±.03	4.05±.01	8.35±.05	10.46±.05
7.40	3.26±.02	4.05±.01	9.00±.00	10.43±.03
7.90	3.21±.00	4.05±.00	9.50±.00	10.58±.06
9.10	3.18±.00	4.04±.01	9.90±.20	9.97±.10

*—analysed on PE-HPLC at 50 µL injection volume
**—no separation of two compounds

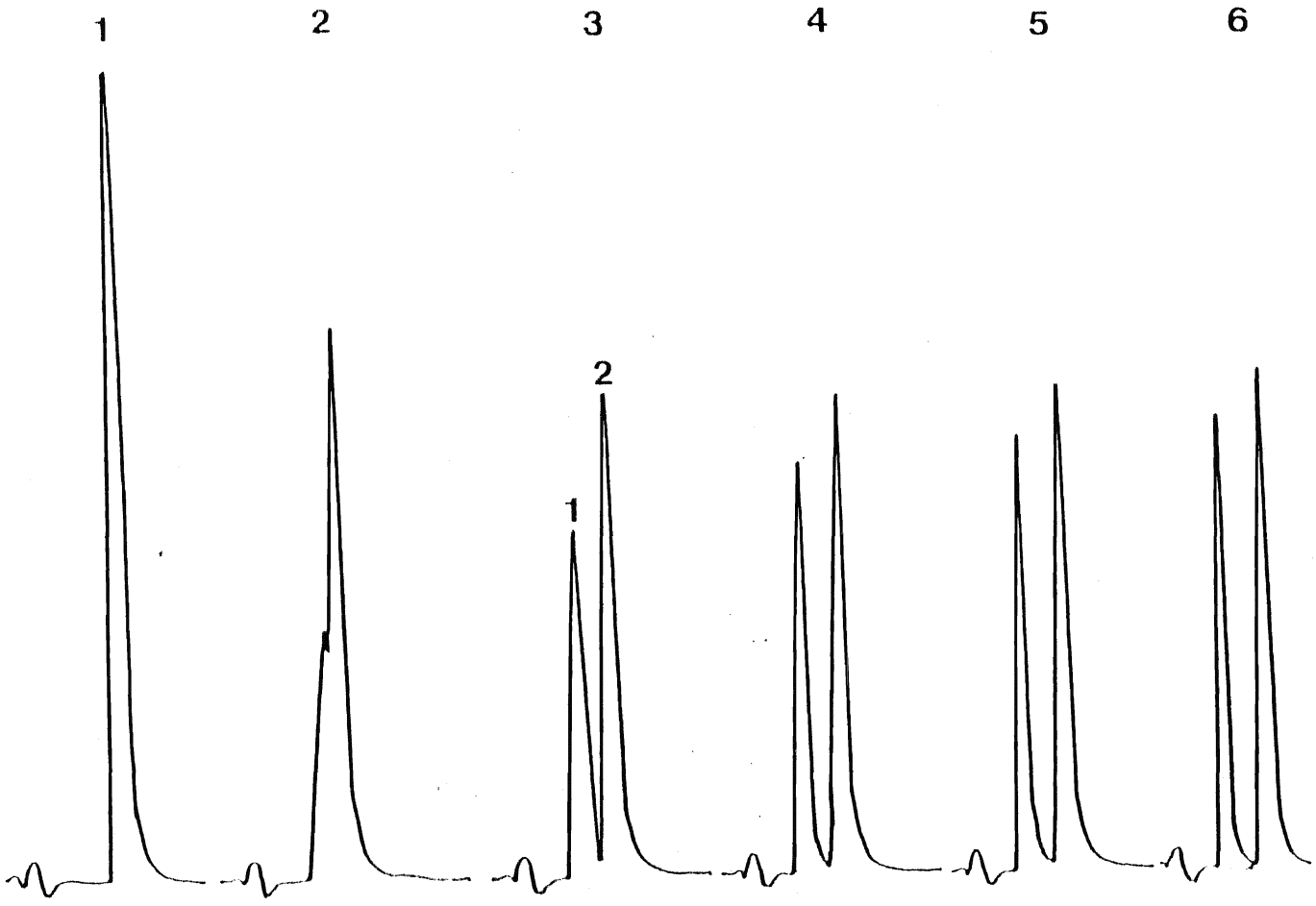


Figure 34: Chromatograms of STB (1) and MBC (2) Showing the Effects of the Sample Solvent pH on the Retention Time and Peak Height of STB. 1) pH 4.42 ; 2) pH 5.95; 3) pH 6.50; 4) pH 6.74; 5) pH 7.40; 6) pH 7.90;

2.Analysis on HP-HPLC

In this study pH was varied from 5 to 8. This range was chosen since buffering capacity of the phosphate buffer at higher and lower pH was poor and had deteriorating effects on the reversed phase column. The concentration of buffer differed from the PE study and was equal to that of the mobile phase of 0.007M.

1) At Constant Mass of Analyte Injected

The complete results are listed in Table XVa for STB and Table XVb for MBC.

Retention Time STB retention time was not influenced by the pH change at 10 and 50 µL injections. As the injection volume increased a successively larger decreases in the retention time of 1.3, and 6.5% were observed for 100, and 200 µL injections as shown in Figure 35. For MBC, pH had no influence on the retention time at any injection volumes (Figure 36).

Peak Height For 10 µL injection, peak height of STB was not influenced by the increase in pH. For higher injection volumes peak heights increased as the pH of sample solution increased from 5 to 8. Increases of 10.7, 37.3, and 25.7% were observed for 50, 100, and 200 µL injections (Figure 37). Peak height for MBC was not affected by the change in the pH (Figure 38).

Peak Width A gradual decrease in the STB peak width was observed as pH was increased (Figure 39). In this study there was marked increase in the peak widths with the increase in

Table XVa

Chromatographic Results for STB when pH of the Sample Solution increases from 5 to 8 and Injected Mass of Analytes Stays Constant with Increasing Injection Volume.

pH OF BUFFER	pH OF SOLUTION	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
			10µL @ 5µg/mL			
5.00	5.61	2.490±.020	87±2	8.87±.25	0.137±.002	0.43±.01
5.00	5.58	2.502±.005	87±2	8.98±.15	0.135±.004	0.43±.01
6.00	6.33	2.494±.008	85±1	8.86±.25	0.134±.004	0.42±.01
6.00	6.37	2.504±.004	87±2	8.90±.24	0.136±.001	0.42±.01
7.00	7.37	2.500±.003	84±1	8.44±.18	0.137±.006	0.44±.02
7.00	7.38	2.498±.003	81±1	8.47±.16	0.135±.003	0.43±.03
8.00	8.27	2.499±.006	85±1	9.01±.15	0.132±.001	0.44±.01
8.00	8.17	2.500±.005	86±1	8.98±.14	0.134±.003	0.43±.01
			50 µL @ 1µg/mL			
5.00	5.44	2.635±.007	91±1	8.91±.15	0.155±.002	0.66±.03
5.00	5.55	2.629±.016	90±2	8.81±.23	0.155±.003	0.61±.04
6.00	6.31	2.634±.003	89±1	9.01±.06	0.146±.008	0.61±.03
6.00	6.36	2.628±.009	89±1	9.01±.11	0.150±.001	0.62±.04
7.00	7.27	2.630±.009	93±1	9.51±.18	0.148±.002	0.63±.04
7.00	7.36	2.627±.002	90±1	9.50±.15	0.144±.004	0.62±.05
8.00	8.17	2.622±.002	91±1	9.77±.18	0.141±.003	0.61±.05
8.00	8.11	2.627±.011	92±2	9.86±.17	0.141±.001	0.64±.04
			100 µL @ .5µg/mL			
5.00	5.59	2.762	93	7.19	0.178	0.92
5.00	5.59	2.763	94	7.21	0.174	0.91
6.00	6.35	2.757	92	7.36	0.164	0.84
6.00	6.35	2.756	92	7.35	0.167	0.96
7.00	7.37	2.735	90	8.37	0.146	0.98
7.00	7.37	2.724	91	8.24	0.151	0.95
8.00	8.22	2.717	94	9.87	0.131	0.88
8.00	8.22	2.739	91	9.91	0.126	0.86
			200µl @ .25µg/ml			
5.00	5.59	2.956	106	7.11	0.201	0.36
5.00	5.59	2.961	105	6.81	0.208	0.33
6.00	6.35	2.908	107	7.09	0.201	0.31
6.00	6.35	2.904	109	7.02	0.204	0.31
7.00	7.37	2.781	107	8.23	0.171	0.25
7.00	7.37	2.785	109	8.33	0.171	0.24
8.00	8.22	2.772*	47	8.81	0.081	0.92
8.00	8.22	2.851*	60	8.49	0.109	0.21
8.00	8.22	2.768	109	8.74	0.164	0.25

* denotes split peaks

Table XVb

Chromatographic Results for MBC when pH of the Sample Solution increases from 5 to 8 and Injected Mass of Analytes Stays Constant with Increasing Injection Volume.

pH OF BUFFER	pH OF SOLUTION	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
			10µL @ 5µg/mL			
5.00	5.61	3.435±.023	110±1	7.65±.30	0.207±.006	0.45±.01
5.00	5.58	3.446±.017	113±3	7.72±.14	0.207±.008	0.44±.01
6.00	6.33	3.441±.019	111±1	7.64±.25	0.207±.008	0.45±.01
6.00	6.37	3.449±.009	112±2	7.68±.25	0.211±.007	0.46±.01
7.00	7.37	3.448±.014	114±1	7.64±.17	0.213±.004	0.44±.01
7.00	7.38	3.448±.017	112±1	7.66±.19	0.212±.009	0.45±.01
8.00	8.27	3.448±.019	106±5	7.47±.13	0.203±.010	0.47±.03
8.00	8.17	3.453±.020	109±3	7.54±.16	0.212±.008	0.45±.01
			50 µL @ 1µg/mL			
5.00	5.44	3.522±.015	117±1	8.83±.17	0.195±.004	0.46±.02
5.00	5.55	3.516±.027	117±3	8.81±.12	0.192±.007	0.46±.01
6.00	6.31	3.524±.012	116±1	8.75±.22	0.191±.006	0.45±.01
6.00	6.36	3.517±.018	115±3	8.75±.23	0.193±.002	0.45±.01
7.00	7.27	3.522±.020	119±1	8.86±.27	0.190±.001	0.45±.02
7.00	7.36	3.524±.019	116±1	8.80±.28	0.189±.001	0.45±.01
8.00	8.17	3.518±.014	115±2	8.76±.28	0.188±.003	0.46±.01
8.00	8.11	3.523±.003	118±1	8.82±.25	0.191±.001	0.45±.01
			100 µL @ .5µg/mL			
5.00	5.59	3.594	119	9.03	0.193	0.53
5.00	5.59	3.593	119	8.94	0.191	0.52
6.00	6.35	3.598	118	8.91	0.191	0.52
6.00	6.35	3.595	115	8.91	0.186	0.53
7.00	7.37	3.595	115	8.89	0.191	0.51
7.00	7.37	3.595	111	8.81	0.192	0.55
8.00	8.22	3.591	115	8.95	0.185	0.52
8.00	8.22	3.615	116	8.98	0.183	0.51
			200µL @ .25µg/mL			
5.00	5.59	3.699	142	9.99	0.215	0.58
5.00	5.59	3.713	149	9.92	0.215	0.65
6.00	6.35	3.709	144	9.86	0.223	0.63
6.00	6.35	3.711	146	9.96	0.223	0.63
7.00	7.37	3.693	139	9.99	0.211	0.64
7.00	7.37	3.699	142	9.95	0.217	0.62
8.00	8.22	3.707	140	9.89	0.213	0.63
8.00	8.22	3.702	141	9.92	0.221	0.62

Figure 35: Plot of Retention Time vs. pH of the Sample Solution for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

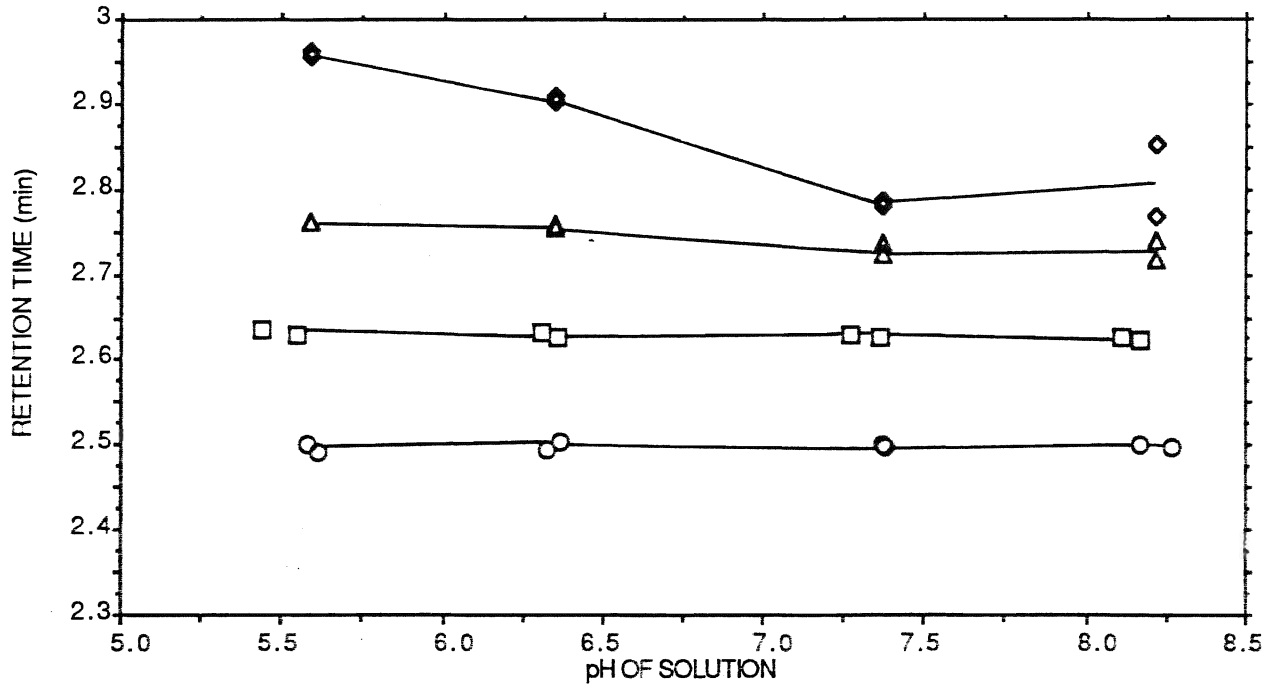


Figure 36: Plot of Retention Time vs. pH of the Sample Solution for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

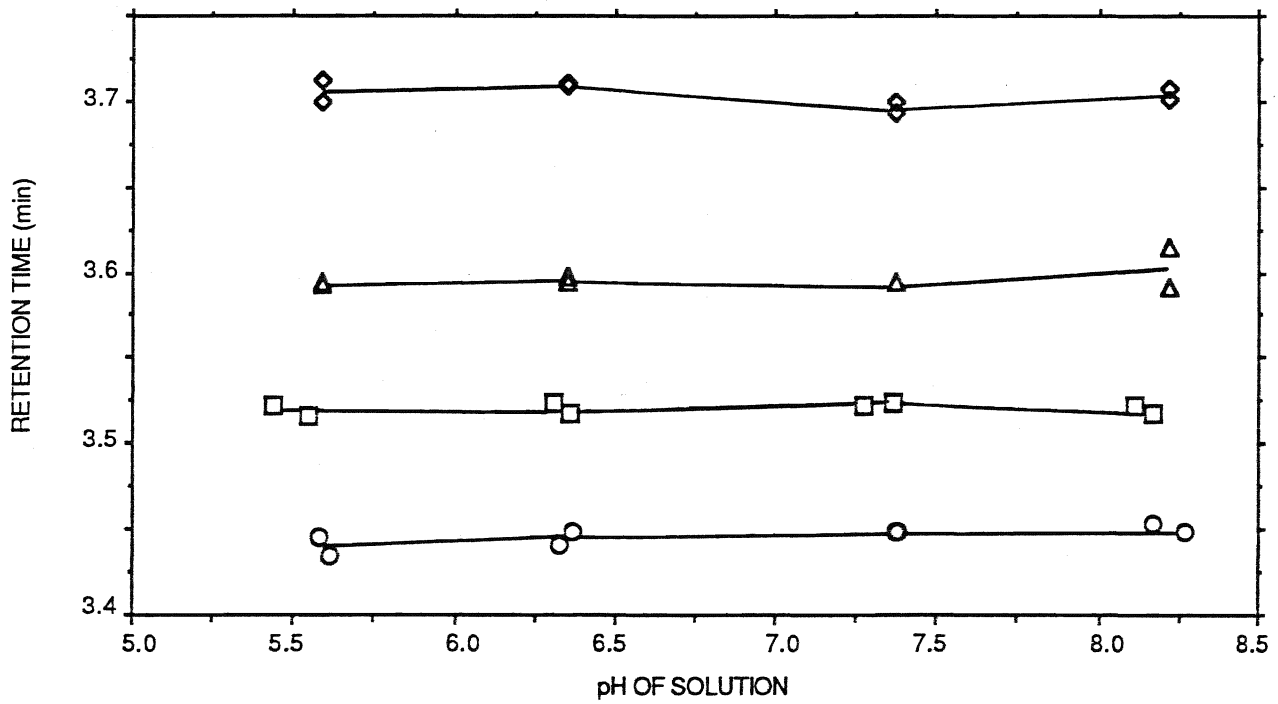


Figure 37: Plot of Peak Height vs. pH of the Sample Solution for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

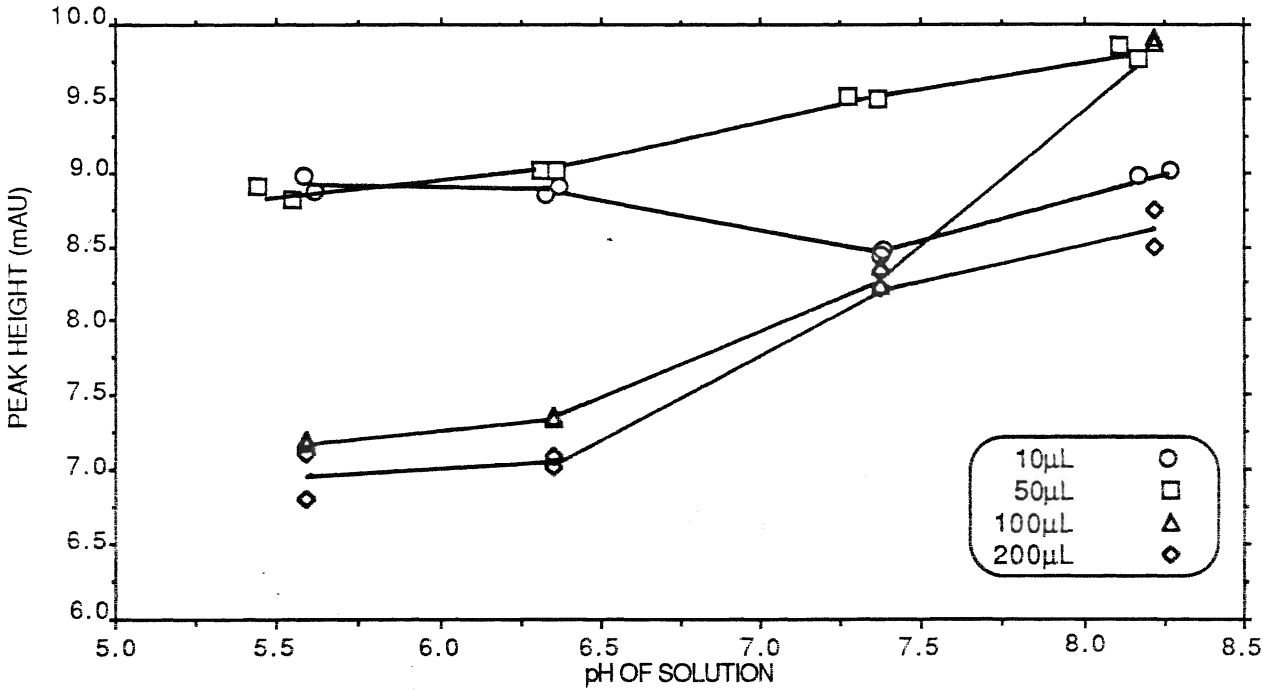


Figure 38: Plot of Peak Height vs. pH of the Sample Solution for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

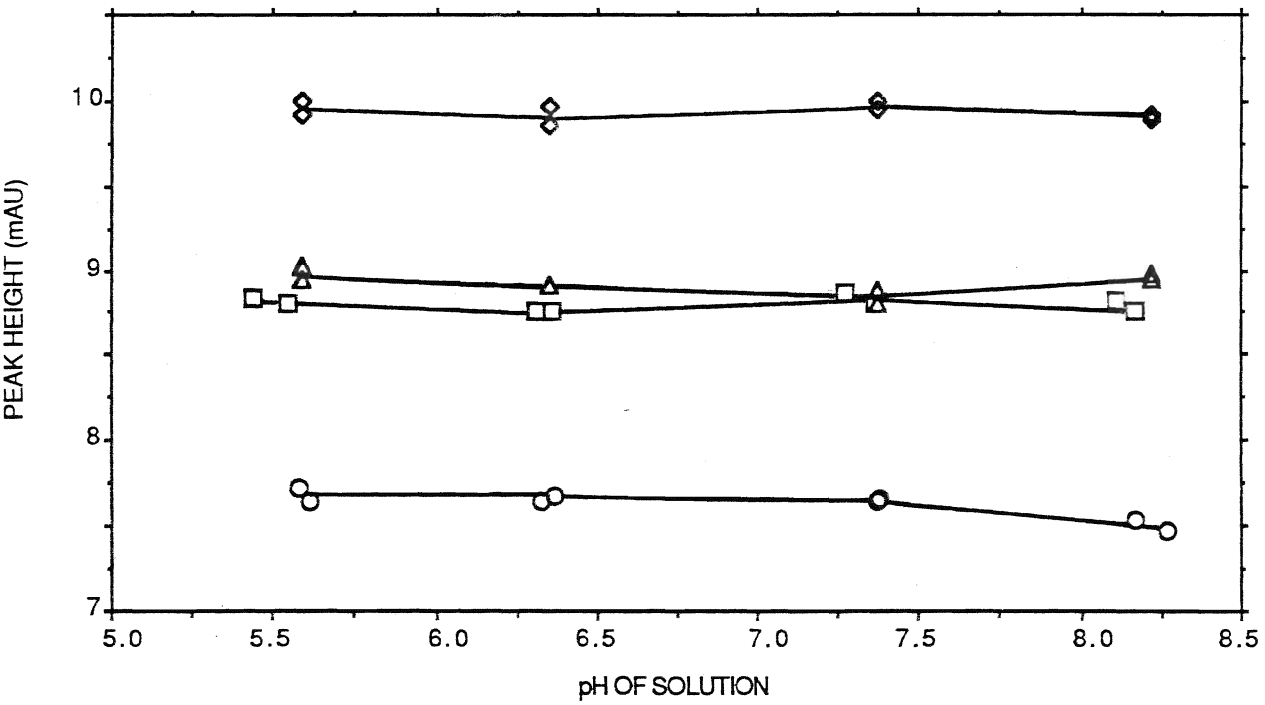


Figure 39: Plot of Peak Width vs. pH of the Sample Solution for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

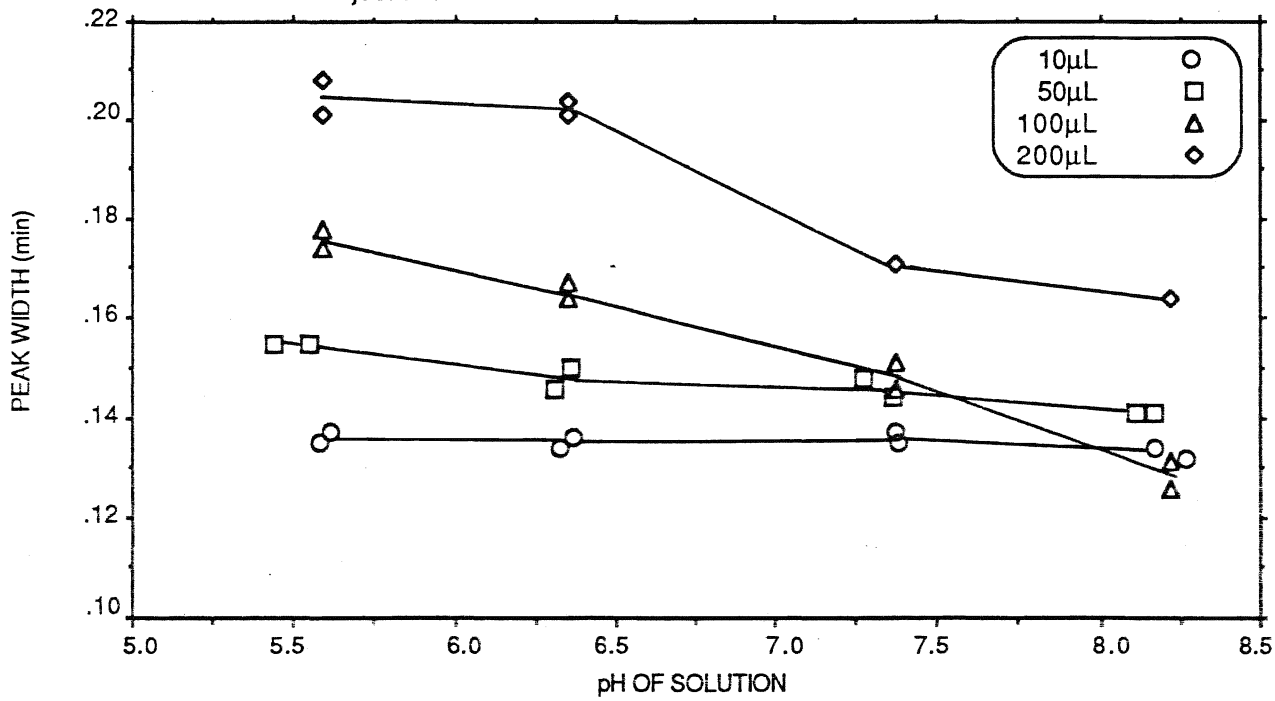
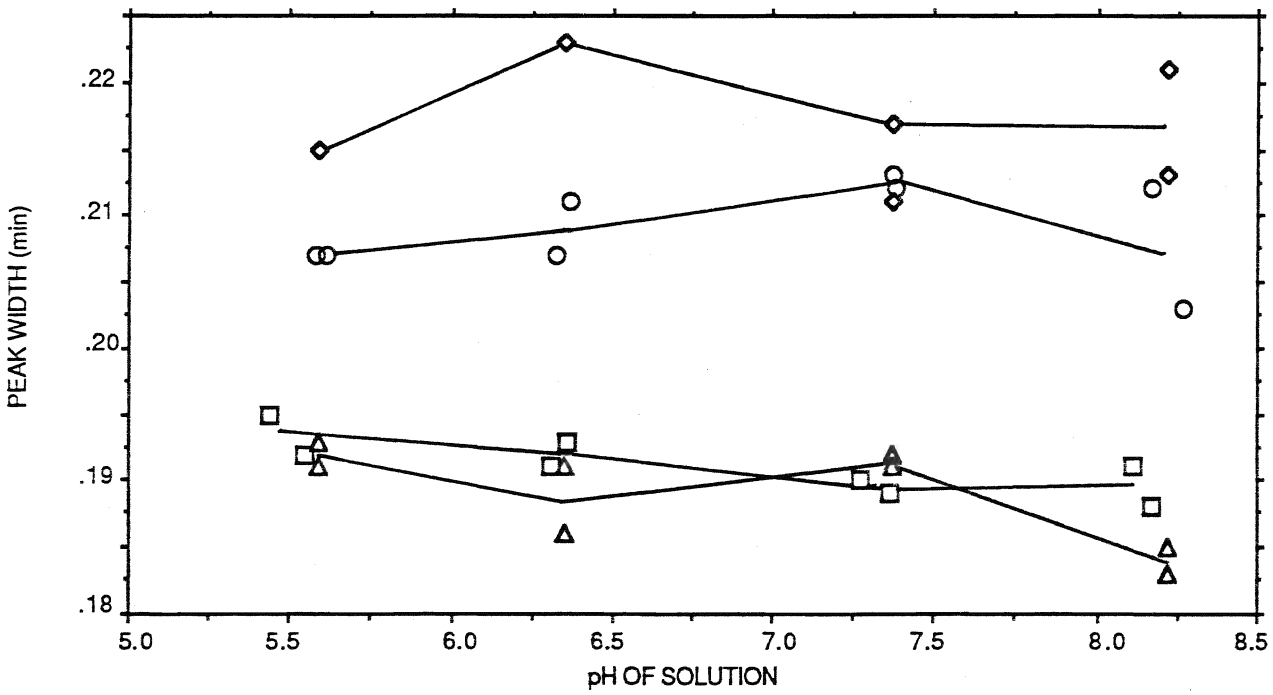


Figure 40: Plot of Peak Width vs. pH of the Sample Solution for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.



the injection volume as can be seen in Figure 39. At pH 5, peak widths were 0.137, 0.155, 0.178, and 0.201 minutes for 10, 50, 100, and 200 µL injections. For MBC, pH had no affect on the peak widths. Also, for all four injection volumes peak widths for MBC were very similar as shown in Figure 40.

Peak Area The change in the pH had no affect on the peak areas of both analytes as can be observed from Tables XV and XVI.

Peak Symmetry The pH influence on the peak symmetry was observed only for STB and only at pH 8 for 100 and 200 µL injections (Table XV).

2) At Increasing Mass of Analyte Injected

The complete results are listed in Tables XVIa and XVIb and the chromatograms are shown in Figure 41.

Retention Time Retention time of STB was not influenced at 10 and 50 µL injections. At 100 and 200 µL injections, decreases of 1.2 and 6.6% were observed. The trend is similar to that in the study at constant mass, but is much smaller than for PE study. Once again the increase in the pH of the sample solution had no influence on the retention time of MBC.

Peak Height At 10 µL injection, pH had no pronounced effect on the peak height of STB. For 50, 100, and 200 µL injections, however, peak heights increased by 11.9, 25.1, and 71.7% (Figure 44). For MBC, the peak heights were not affected by the increase in the pH at any of the four injection volumes (Figur 45).

Table XVIa

Chromatographic Results for STB when pH of the Sample Solution increases from 5 to 8 and Injected Mass of Analytes Increases with Increasing Injection Volume.

pH OF BUFFER	pH OF SOLUTION	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
			10µL @ 5µg/mL			
5.00	5.61	2.499±.002	87±2	8.81±.25	0.137±.002	0.43±.01
5.00	5.58	2.502±.005	87±2	8.98±.15	0.135±.004	0.43±.01
6.00	6.33	2.494±.008	85±1	8.86±.25	0.134±.004	0.42±.01
6.00	6.37	2.504±.004	87±2	8.90±.24	0.136±.001	0.42±.01
7.00	7.37	2.500±.003	84±1	8.44±.18	0.137±.006	0.44±.02
7.00	7.38	2.498±.003	81±1	8.47±.16	0.135±.003	0.43±.03
8.00	8.27	2.499±.006	85±1	9.01±.15	0.132±.001	0.44±.01
8.00	8.17	2.500±.005	86±1	8.98±.14	0.134±.003	0.43±.01
			50 µL @ 5µg/mL			
5.00	5.61	2.632	93	9.16	0.153	0.59
5.00	5.58	2.637	93	9.14	0.152	0.59
6.00	6.33	2.638	92	9.23	0.151	0.59
6.00	6.37	2.638	92	9.11	0.151	0.61
7.00	7.37	2.638	86	9.19	0.141	0.62
7.00	7.38	2.639	86	9.22	0.141	0.65
8.00	8.27	2.644	91	10.25	0.136	0.66
8.00	8.17	2.633	91	10.23	0.135	0.64
			100 µl @ 5µg/ml			
5.00	5.61	2.744	93	9.63	0.141	0.52
5.00	5.58	2.748	93	9.68	0.138	0.52
6.00	6.33	2.742	92	9.89	0.134	0.51
6.00	6.37	2.749	92	9.91	0.133	0.52
7.00	7.37	2.728	86	10.59	0.116	0.52
7.00	7.38	2.736	88	10.69	0.117	0.51
8.00	8.27	2.714	93	12.09	0.109	0.54
8.00	8.17	2.711	92	12.05	0.109	0.53
			200 µL @ 5µg/mL			
5.00	5.61	3.054	92	7.30	0.194	0.74
5.00	5.58	3.062	92	7.20	0.186	0.74
6.00	6.33	3.005	91	7.80	0.167	0.71
6.00	6.37	2.998	92	7.80	0.169	0.71
7.00	7.37	2.879	87	10.40	0.118	0.55
7.00	7.38	2.871	86	10.50	0.119	0.56
8.00	8.27	2.854	93	12.50	0.106	0.39
8.00	8.17	2.854	93	12.40	0.107	0.56

Table XVIb

Chromatographic Results for MBC when pH of the Sample Solution increases from 5 to 8 and Injected Mass of Analytes Increases with Increasing Injection Volume.

pH OF BUFFER	pH OF SOLUTION	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
			10µL @ 5µg/mL			
5.00	5.61	3.435±.023	110±1	7.65±.30	0.207±.006	0.45±.01
5.00	5.58	3.446±.017	113±3	7.72±.14	0.207±.008	0.44±.01
6.00	6.33	3.441±.019	111±1	7.64±.25	0.207±.008	0.45±.01
6.00	6.37	3.449±.009	112±2	7.68±.25	0.211±.007	0.46±.01
7.00	7.37	3.448±.014	114±1	7.64±.17	0.213±.004	0.44±.01
7.00	7.38	3.448±.017	112±1	7.66±.19	0.212±.009	0.45±.01
8.00	8.27	3.448±.019	106±5	7.47±.13	0.203±.010	0.47±.03
8.00	8.17	3.453±.020	109±3	7.54±.16	0.212±.008	0.45±.01
			50 µL @ 5µg/mL			
5.00	5.61	3.532	119	8.47	0.205	0.41
5.00	5.58	3.518	120	8.44	0.206	0.39
6.00	6.33	3.524	123	8.49	0.209	0.38
6.00	6.37	3.519	122	8.46	0.209	0.38
7.00	7.37	3.521	119	8.42	0.206	0.39
7.00	7.38	3.527	111	8.38	0.205	0.41
8.00	8.27	3.532	116	8.32	0.202	0.41
8.00	8.17	3.523	116	8.28	0.207	0.41
			100 µL @ 5µg/mL			
5.00	5.61	3.583	125	8.83	0.205	0.39
5.00	5.58	3.584	124	8.83	0.202	0.37
6.00	6.33	3.586	124	8.82	0.202	0.51
6.00	6.37	3.595	121	8.77	0.201	0.39
7.00	7.37	3.594	119	8.73	0.198	0.39
7.00	7.38	3.603	124	8.78	0.203	0.39
8.00	8.27	3.585	118	8.64	0.197	0.41
8.00	8.17	3.582	117	8.64	0.197	0.41
			200 µL @ 5µg/mL			
5.00	5.61	3.707	124	9.20	0.194	0.41
5.00	5.58	3.704	124	9.20	0.194	0.41
6.00	6.33	3.703	123	9.10	0.194	0.41
6.00	6.37	3.696	123	9.20	0.194	0.41
7.00	7.37	3.706	124	9.10	0.195	0.39
7.00	7.38	3.698	124	9.20	0.195	0.39
8.00	8.27	3.698	121	9.10	0.193	0.39
8.00	8.17	3.699	121	9.10	0.194	0.39

Figure 41: Chromatograms of STB (1) and MBC (2) for Increasing Mass Study Showing the Effects of the Sample Solvent pH at increasing injection Volumes. 1) pH 5.0; 2) pH 6.0; 3) pH 7.0; 4) pH 8.0.

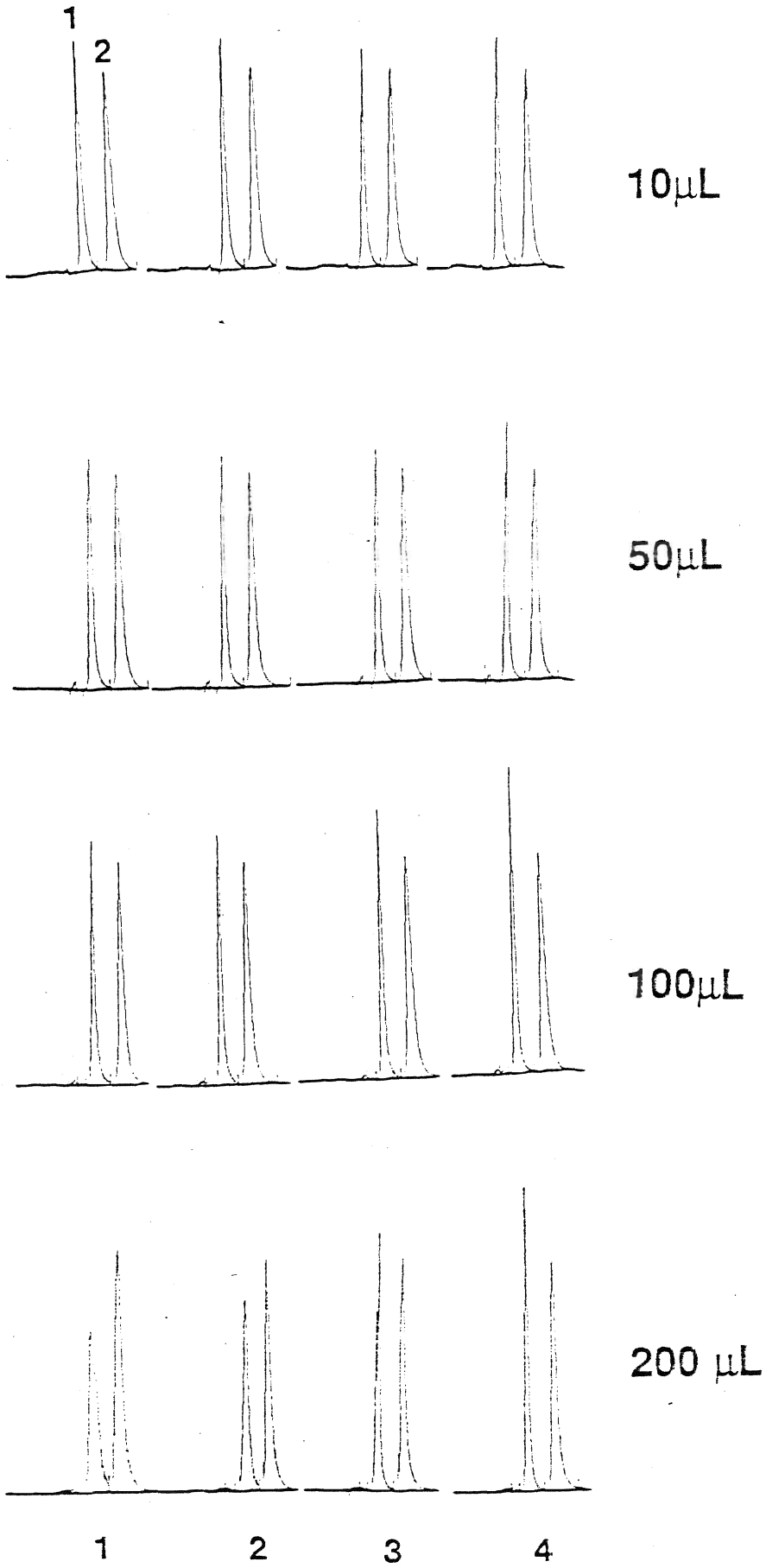


Figure 42: Plot of Retention Time vs. pH of the Sample Solution for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

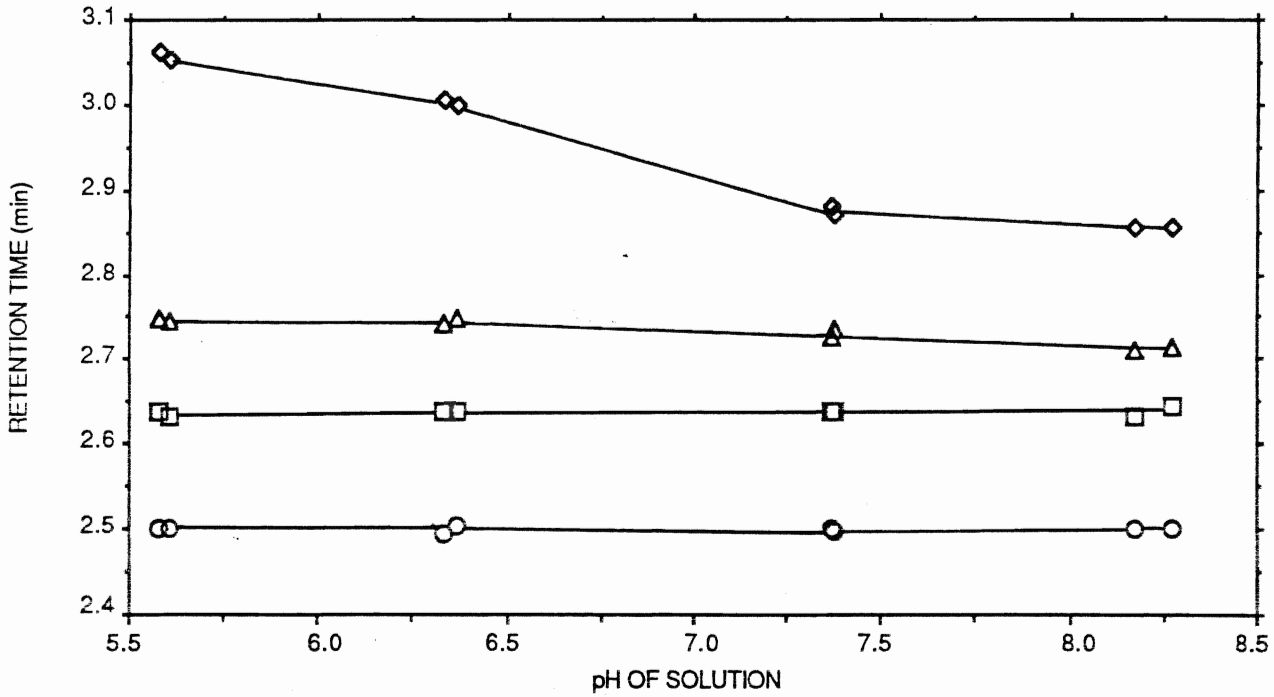


Figure 43: Plot of Retention Time vs. pH of the Sample Solution for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

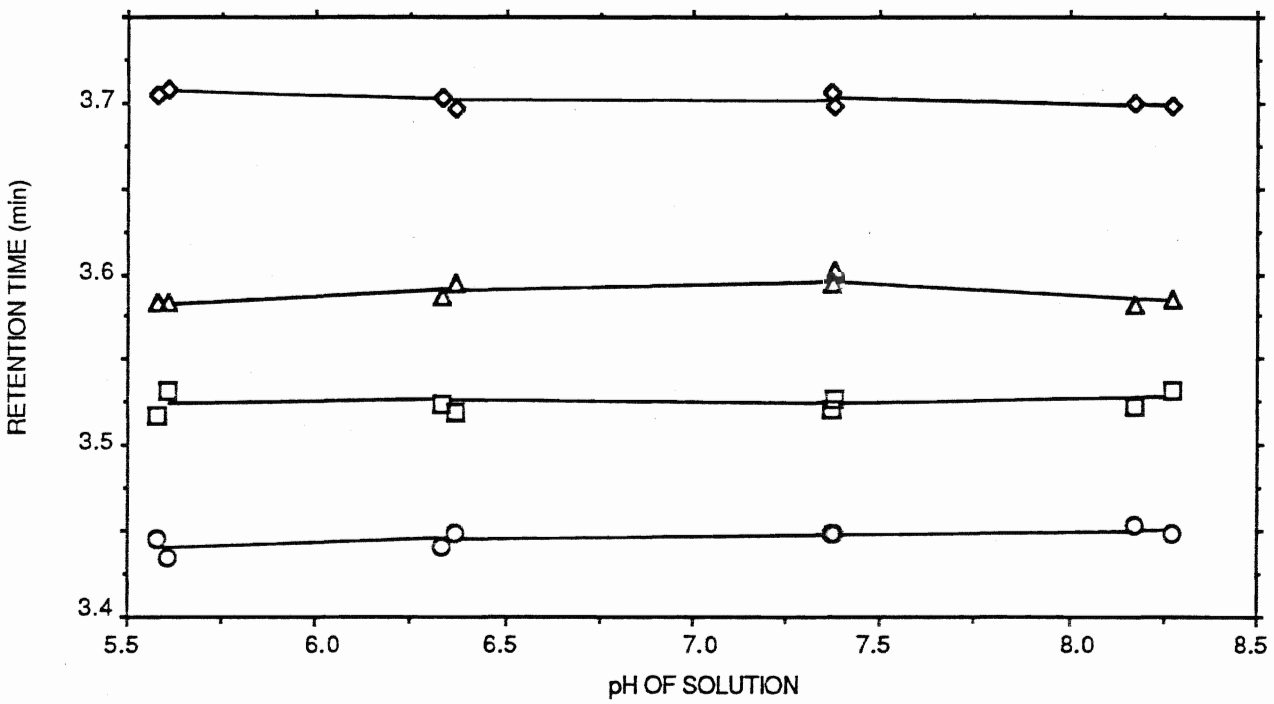


Figure 44: Plot of Peak Height vs. pH of the Sample Solution for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

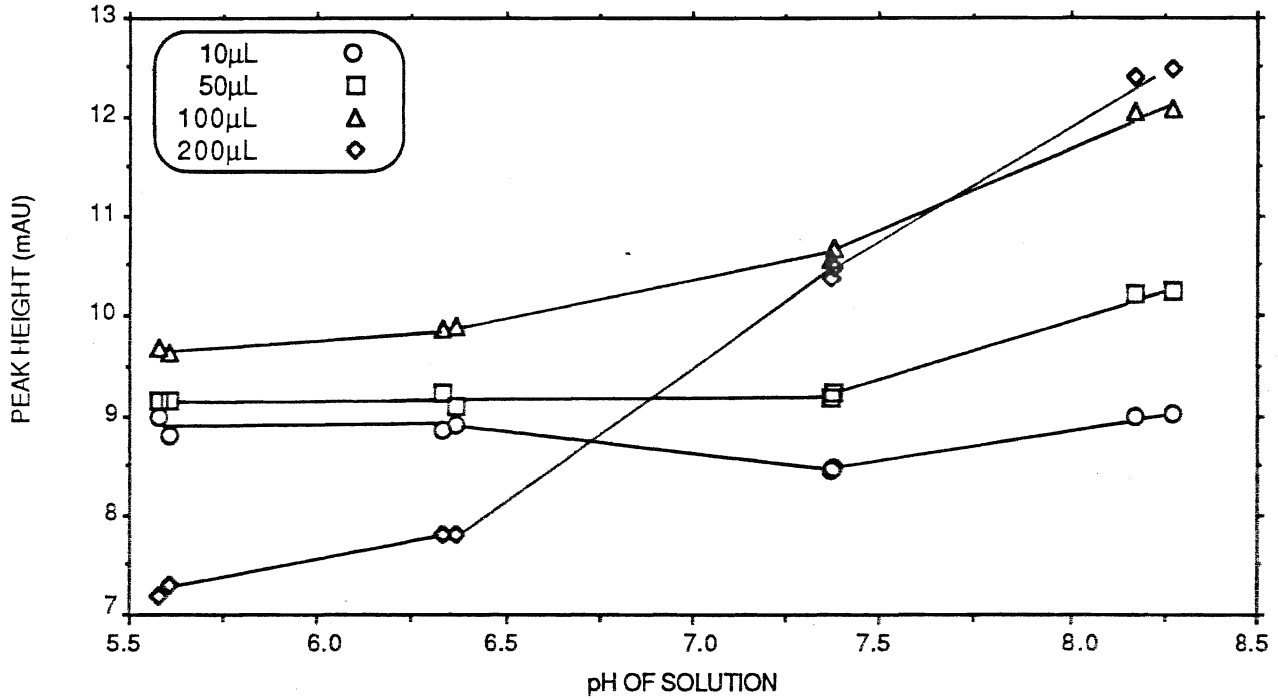


Figure 45: Plot of Peak Height vs. pH of the Sample Solution for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

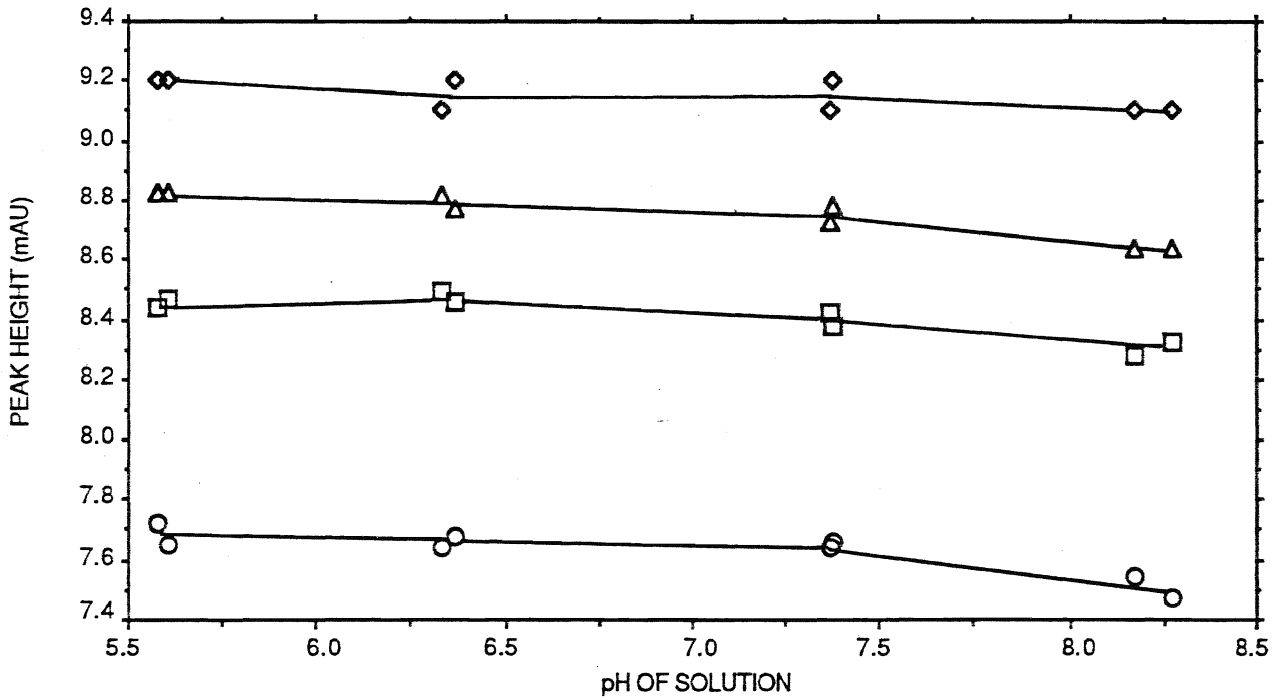


Figure 46: Plot of Peak Width vs. pH of the Sample Solution for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

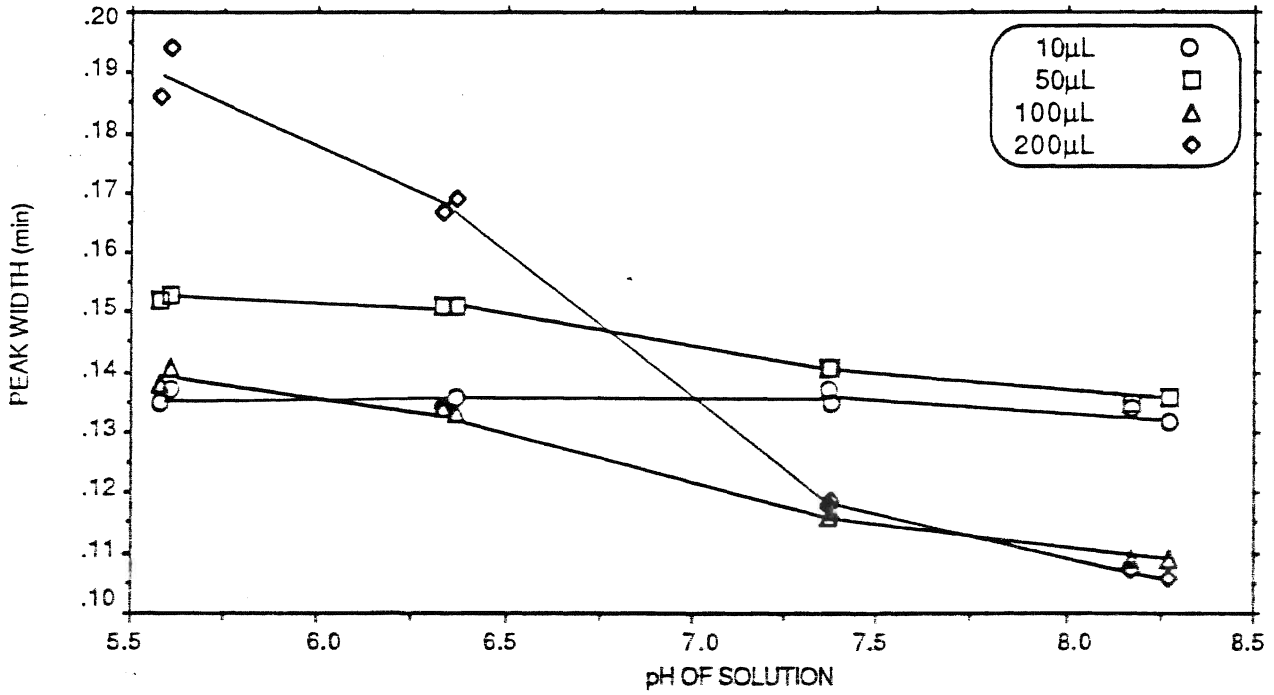
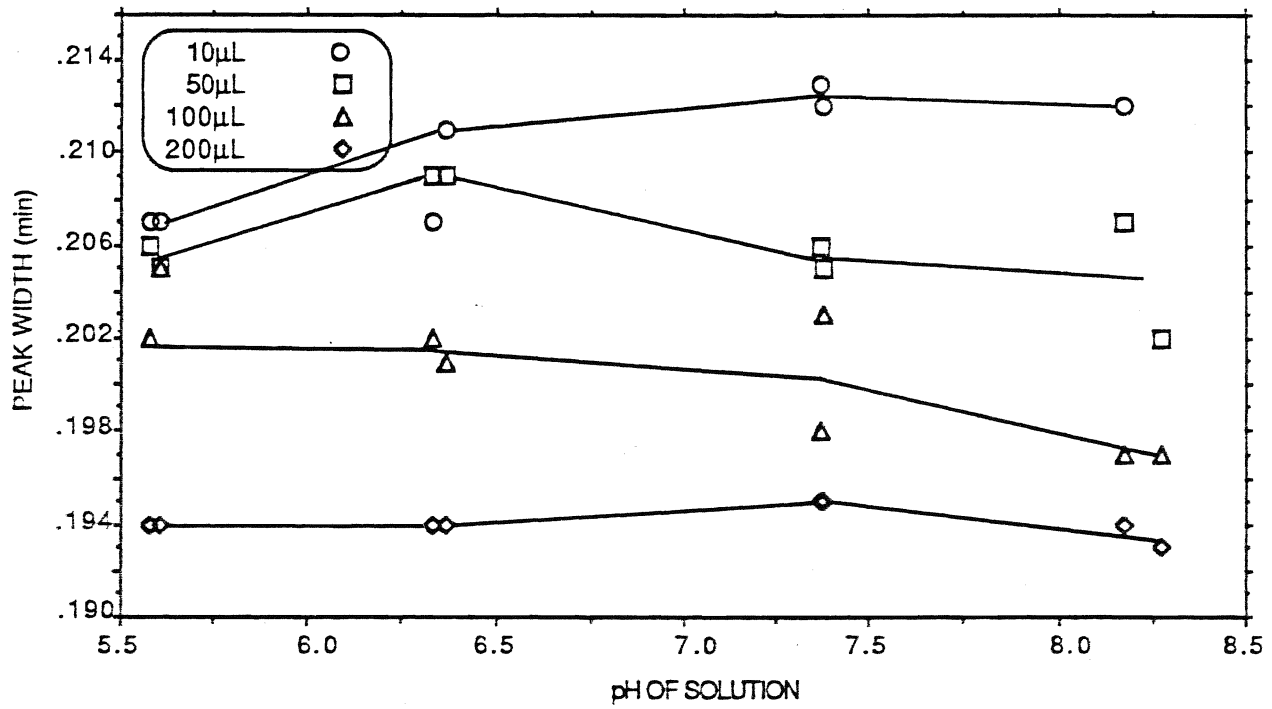


Figure 47: Plot of Peak Width vs. pH of the Sample Solution for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.



Peak Width As for constant mass study, here also peak widths of STB decreased with the pH increase. The decreases became larger as the volume of the injection increased. Decreases of 2.2, 11.1, 21.5, and 43.9% were observed (Figure 46). It was clear that pH had no pronounced effect on the MBC peak width (Figure 47).

Peak Area The peak areas of both analytes were not influenced by the increase in the pH of the sample solutions (Tables XVIa and XVIb).

Peak Symmetry The symmetry of both peaks was also not affected by the pH increase.

D. Effect of Buffer Concentration in the Sample Solution on the Chromatographic Peak Profile

1. Analysis on PE-HPLC

In this experiment buffer concentration was varied from 0 to 0.14 M and the acetonitrile concentration was set to 35%. Results were obtained at 10 and 50 µL injection volumes. The complete results are listed in Table XVII.

Retention Time Buffer concentration had no apparent effect on the retention time of STB at 10 µL injection. At 50 µL injection, a decrease of 5.8% was observed but only for highest buffer concentration. For MBC, a minor reduction of 2.2% was observed at the highest concentration of buffer with

Table XVII*

Influence of Buffer Concentration on the Peak Profile of STB and MBC

[Buffer]	Retention Time (min)		Peak Height (cm)	
M	STB	MBC	STB	MBC
		10 µL		
0.0000	3.18±.02	3.92±.02	11.71±.11	16.42±.10
0.0007	3.24±.00	3.92±.00	11.99±.16	16.43±.25
0.0070	3.20±.00	3.90±.00	12.40±.00	16.61±.20
0.0700	3.20±.01	3.92±.02	12.99±.13	16.69±.09
0.1400	3.20±.01	3.90±.01	13.10±.17	16.72±.18
		50 µL		
0.0000	2.98±.00	3.74±.03	7.65±.09	11.74±.21
0.0000	3.00±.00	3.70±.00	7.70±.00	11.62±.42
0.0007	2.98±.01	3.72±.02	8.13±.05	12.07±.03
0.0007	2.98±.01	3.70±.02	7.92±.10	11.84±.19
0.0070	3.00±.00	3.68±.02	9.12±.03	12.17±.03
0.0070	3.00±.00	3.68±.02	8.98±.03	12.02±.08
0.0700	3.00±.00	3.70±.03	9.49±.34	11.82±.11
0.0700	2.98±.01	3.66±.02	9.54±.23	11.68±.31
0.1400	2.80±.00	3.64±.00	10.79±.14	11.83±.17
0.1400	2.84±.00	3.64±.00	10.75±.15	11.77±.12

*—analysed on PE-HPLC

the 50 uL injection.

Peak Height An increase in the peak heights was observed with buffer concentration increase. For 10 µL injection, peak height increased by 11.9% and at 50 µl injection, increase was 40.7%. There was no influence on the MBC peak heights.

2. Analysis on HP-HPLC

In this study buffer concentration was varied from 0 to 0.12 M and acetonitrile concentration was set at 5%.

1) At Constant Mass of Analyte Injected

The complete results are listed in Table XVIIIa for STB and Table XVIIIb for MBC. To eliminate any possibility of a systematic influence due to the instrument, an injection sequence was set up with one injection for each buffer concentration at each volume studied. The complete sequence was repeated three times. Since the average values were obtained with the values of 3 injections which were not made consecutively, but were made at different times, a larger injection errors were obtained, but the observed trends were reproducible. It should be pointed out that the reproducibility of MBC measurements in the same analyses was much better than for STB as can be observed from Tables XVIIIa and XVIIIb.

Retention Time Increases in STB retention times of 2.5, 2.3, and 1.4% were observed as buffer concentration increased

Table XVIIIa

Chromatographic Results for STB when [Buffer] in the Sample Solvent increases from 0 to 0.12M and Injected Mass of Analytes Stays Constant at Increasing Injection Volume.

[BUFFER] M	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10µL @ 5µg/mL			
0.00	2.248±.017	80±1	9.35±.14	0.121±.001	0.37±.01
0.00	2.250±.004	84±6	9.57±.14	0.122±.006	0.35±.02
0.0007	2.254±.002	82±1	9.50±.03	0.118±.002	0.36±.02
0.0007	2.254±.003	82±4	9.18±.06	0.123±.004	0.35±.01
0.007	2.260±.004	85±1	10.13±.13	0.117±.001	0.34±.02
0.007	2.263±.001	86±2	10.08±.02	0.118±.003	0.36±.01
0.07	2.293±.007	89±2	11.15±.25	0.112±.001	0.35±.02
0.07	2.288±.004	84±1	11.07±.29	0.112±.003	0.37±.02
0.12	2.306±.003	89±4	11.30±.33	0.110± 0	0.37±.02
0.12	2.306±.005	88±1	11.34±.17	0.109±.002	0.37±.02
		50 µL @ 1µg/mL			
0.00	2.345±.006	92±5	12.71±.55	0.101±.001	0.37±.02
0.00	2.343±.001	88±4	12.27±.20	0.100±.005	0.36±.01
0.0007	2.339±.001	91±5	12.51±.26	0.101±.005	0.37±.02
0.0007	2.341±.004	91±2	12.49±.51	0.101±.006	0.36±.03
0.007	2.352±.002	94±2	12.37±.56	0.104±.003	0.37±.03
0.007	2.349± 0	92±3	12.19±.60	0.104±.002	0.34±.01
0.07	2.385±.001	91±1	11.84±.30	0.105±.002	0.34±.01
0.07	2.388±.003	92±2	11.85±.10	0.107±.003	0.34±.03
0.12	2.399±.004	92±1	11.95±.36	0.106±.002	0.34±.02
0.12	2.396±.003	91±1	11.83±.30	0.105±.002	0.35±.02
		100µl @ .5µg/ml			
0.00	2.426±.001	93±4	12.11±.33	0.105±.007	0.39±.01
0.00	2.427± 0	97±4	12.04±.24	0.110±.005	0.38±.02
0.0007	2.427±.001	97±1	12.00±.47	0.111±.002	0.36±.03
0.0007	2.430±.005	96±1	12.03±.40	0.110±.005	0.37±.01
0.007	2.434±.001	93±4	11.70±.30	0.109±.007	0.38±.01
0.007	2.435±.003	94±1	11.69±.40	0.111±.004	0.37±.01
0.07	2.465±.001	101±4	11.69±.34	0.117±.009	0.34±.01
0.07	2.456±.008	98±6	11.46±.13	0.117±.008	0.34±.01
0.12	2.462±.001	96 ±1	12.00±.46	0.109±.004	0.36±.01
0.12	2.461±.005	94±2	11.50±.57	0.175±.006	0.35±.01
		200µL @ .25µg/mL			
0.00	2.557±.001	112±2	12.86± .83	0.120±.010	0.40±.01
0.00	2.556±.001	112±1	12.68±1.09	0.123±.013	0.42±.05
0.0007	2.556±.008	109±3	12.33±1.10	0.125±.010	0.37±.03
0.0007	2.559±.008	112±1	12.29±1.44	0.129±.029	0.39±.02
0.007	2.563±.002	111±1	11.92±1.10	0.131±.016	0.38±.01
0.007	5.565±.003	110±3	11.94±1.26	0.129±.013	0.37±.01
0.07	2.559±.009	115±2	12.62±1.50	0.126±.019	0.33±.02
0.07	2.557±.005	116±2	12.58±1.40	0.127±.016	0.32±.02
0.12	2.537±.005	120±7	12.46±1.40	0.134±.021	0.33±.06
0.12	2.450±.008	107±1	11.20±1.00	0.132±.020	0.32±.06

Table XVIIIb

Chromatographic Results for MBC when [Buffer] in the Sample Solvent increases from 0 to 0.12M and Injected Mass of Analytes Stays Constant at Increasing Injection Volume.

[BUFFER] M	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10µL @ 5µg/mL			
0.00	3.310±.020	109±1	7.82±.17	0.203±.004	0.38±.02
0.00	3.320±.020	109±2	7.85±.14	0.198±.001	0.39±.02
0.0007	3.314± 0	111±1	8.01±.01	0.198±.003	0.39±.02
0.0007	3.315±.001	113±2	8.02±.09	0.200±.001	0.40±.01
0.007	3.322±.004	112±2	7.87±.07	0.205±.008	0.39±.01
0.007	3.323±.002	110±1	7.83±.08	0.205±.002	0.39±.01
0.07	3.323±.001	111±1	7.89±.04	0.204±.006	0.39±.01
0.07	3.318±.001	112±3	7.84±.08	0.210±.002	0.38±.01
0.12	3.320±.002	112±2	7.85±.05	0.206±.004	0.39±.01
0.12	3.322±.003	111±3	7.88±.17	0.205±.006	0.38±.02
		50 µL @ 1µg/mL			
0.00	3.425± 0	119±0	8.93±.10	0.192±.008	0.37±.02
0.00	3.421±.005	117±1	8.81±.01	0.183±.002	0.37±.01
0.0007	3.419±.003	118±1	8.87±.04	0.190±.003	0.36±.01
0.0007	3.422±.002	118±1	8.81±.05	0.188±.001	0.36±.01
0.007	3.426±.002	117±2	8.80±.03	0.189± 0	0.36±.01
0.007	3.417±.007	117±2	8.79±.05	0.191±.004	0.36±.02
0.07	3.419±.003	120±2	8.89±.02	0.188±.003	0.36±.01
0.07	3.420±.001	117±1	8.87±.02	0.191±.004	0.38±.01
0.12	3.412±.001	118±1	8.96±.01	0.184±.001	0.37±.01
0.12	3.405±.007	116±3	8.98±.01	0.186±.005	0.37±.03
		100µL @ .5µg/mL			
0.00	3.490±.007	119±2	8.99±.01	0.188±.001	0.37±.01
0.00	3.492±.007	117±2	8.90±.06	0.188±.001	0.37±.02
0.0007	3.491±.003	117±1	8.82±.12	0.190± 0	0.37±.01
0.0007	3.494±.010	118±1	8.89±.08	0.193±.002	0.36±.01
0.007	3.490±.009	119±1	8.87±.05	0.192±.007	0.36±.01
0.007	3.493±.004	117±7	8.85±.06	0.197±.004	0.36±.03
0.07	3.480±.005	120±2	9.04±.01	0.192±.004	0.36±.01
0.07	3.473±.003	122±3	9.11±.01	0.195±.004	0.36±.01
0.12	3.459±.003	115±1	9.15±.13	0.186±.004	0.36±.01
0.12	3.459±.002	109±2	8.91±.15	0.175±.006	0.39±.02
		200µL @ .25µg/mL			
0.00	3.602±.010	143±1	10.68±.15	0.196±.002	0.35±.01
0.00	3.598±.009	143±2	10.51±.17	0.196±.009	0.35±.01
0.0007	3.602±.005	139±3	10.34±.24	0.195±.004	0.36±.01
0.0007	3.605±.015	140±1	10.27±.29	0.199±.012	0.36±.01
0.007	3.600±.006	144±1	10.46±.28	0.196±.009	0.35±.01
0.007	3.605±.008	142±2	10.40±.36	0.196±.003	0.35±.01
0.07	3.583±.009	144±1	10.61±.38	0.194±.006	0.35±.01
0.07	3.581±.009	141±2	10.62±.33	0.196±.006	0.35±.01
0.12	3.563±.002	143±5	10.78±.29	0.194±.010	0.39±.04
0.12	3.563±.009	132±1	9.93±.44	0.195±.010	0.35±.01

Figure 48: Chromatograms of STB (1) and MBC (2) for Constant Mass Study Showing the Effects of Increasing Buffer Concentration in the Sample Solvent: 1) 0.0M; 2) 0.0007M; 3) 0.007M; 4) 0.07M; 5) 0.12M.

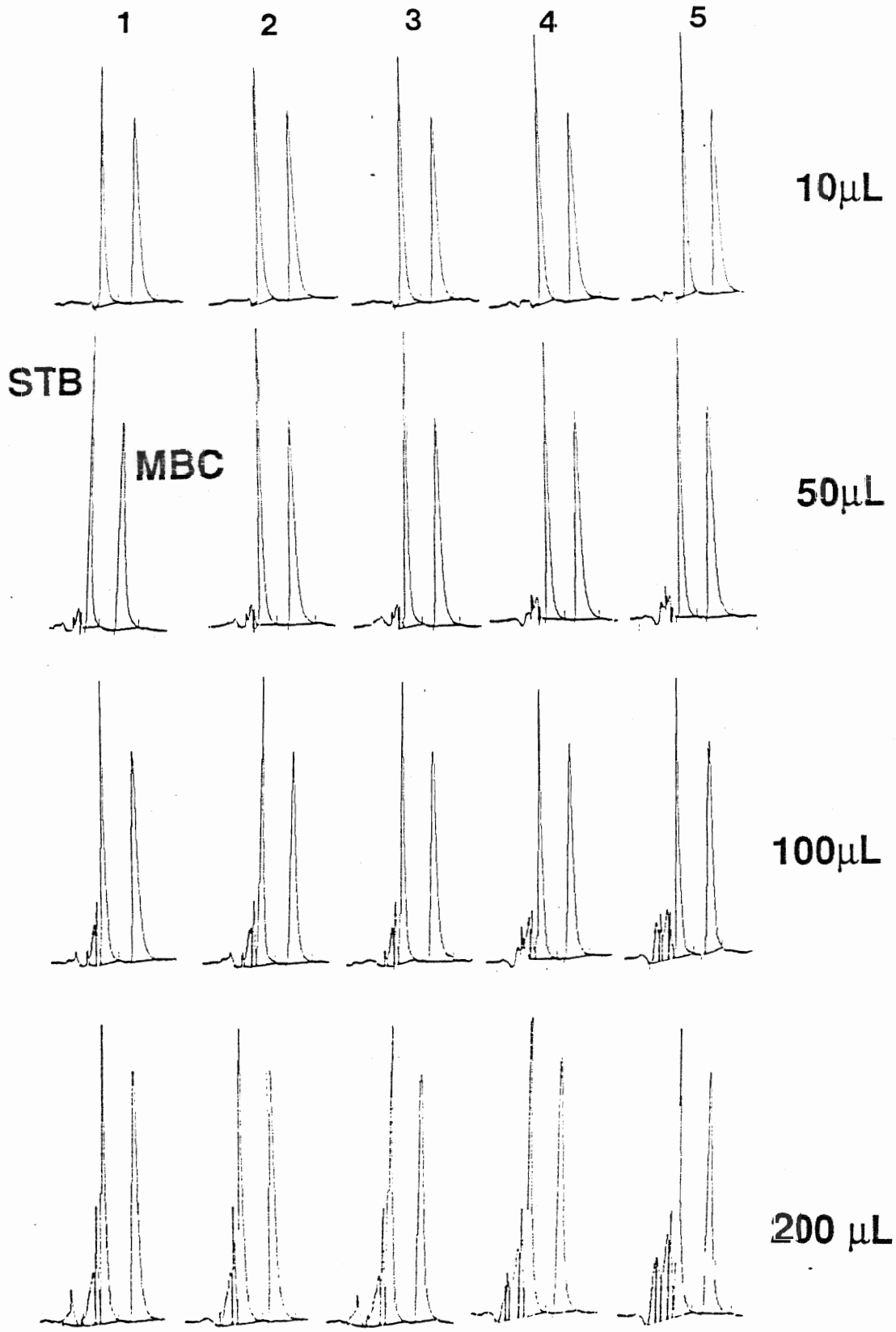


Figure 49: Plot of Retention Time vs. log[Buffer Concentration] for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

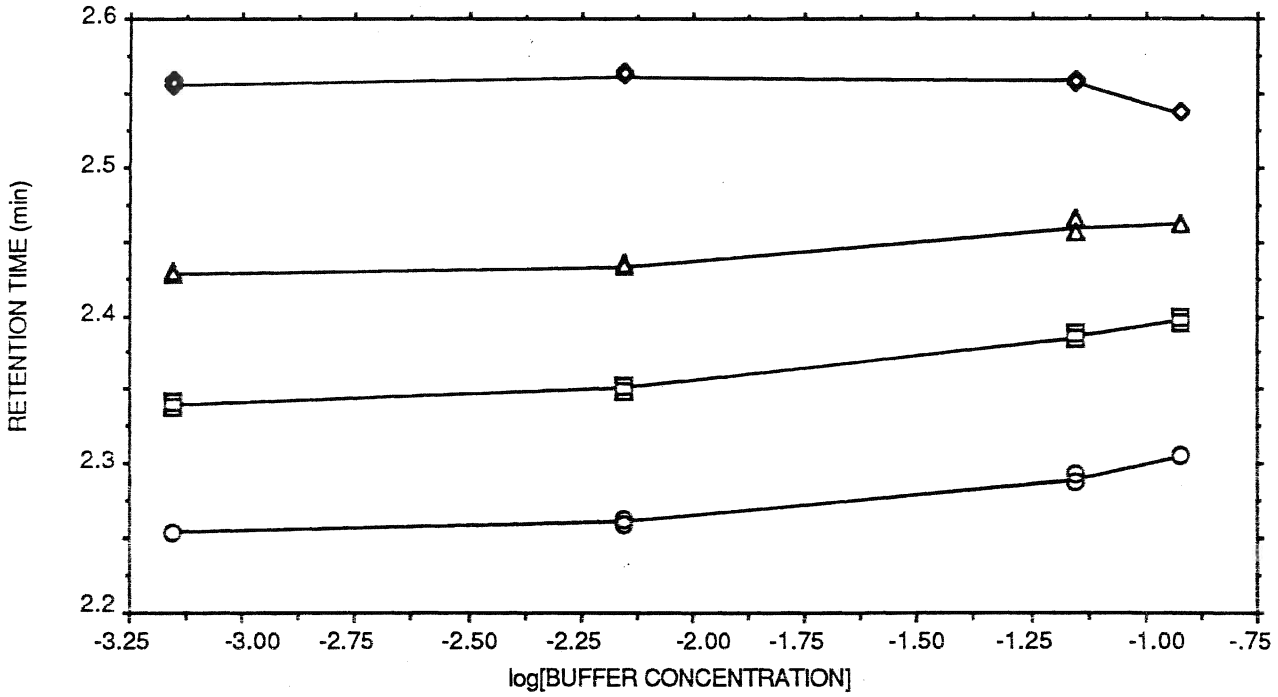


Figure 50: Plot of Retention Time vs. log[Buffer Concentration] for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

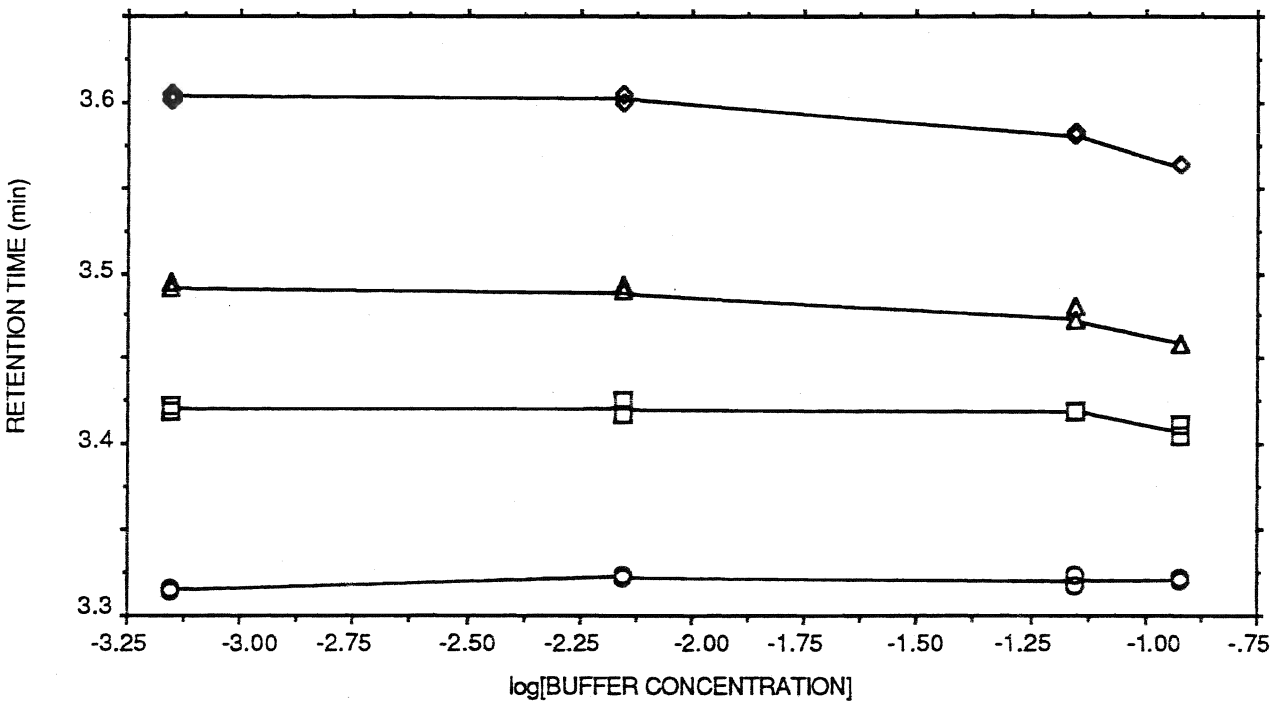


Figure 51: Plot of Peak Height vs. log[Buffer Concentration] for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

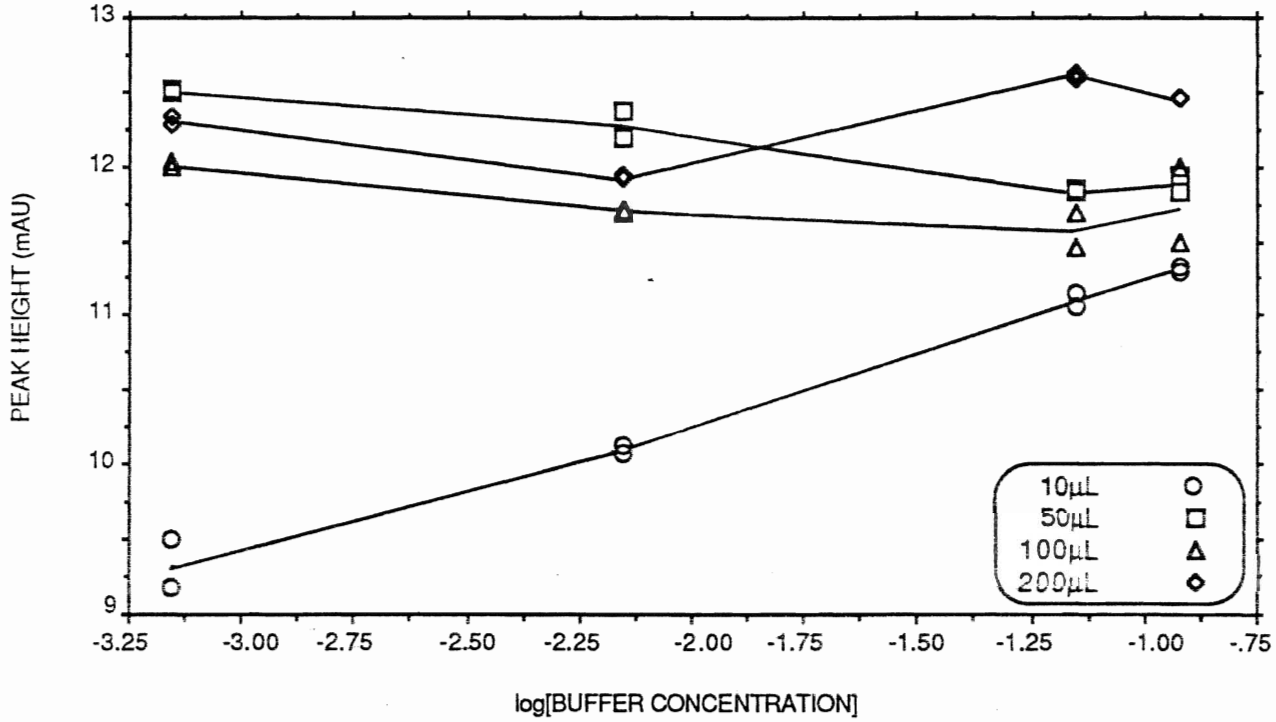


Figure 52: Plot of Peak Height vs. log[Buffer Concentration] for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.

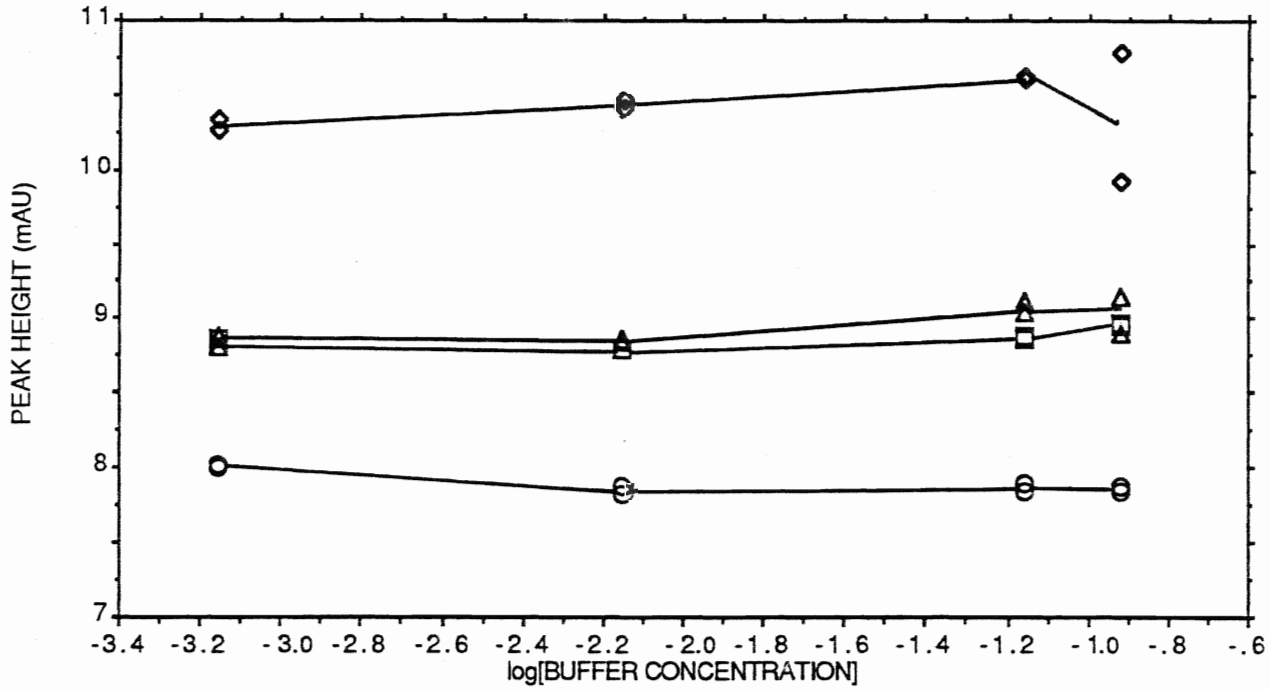


Figure 53: Plot of Peak Width vs. log[Buffer Concentration] for STB at Constant Analyte Mass for 10, 50, 100 and 200 µL Injections.

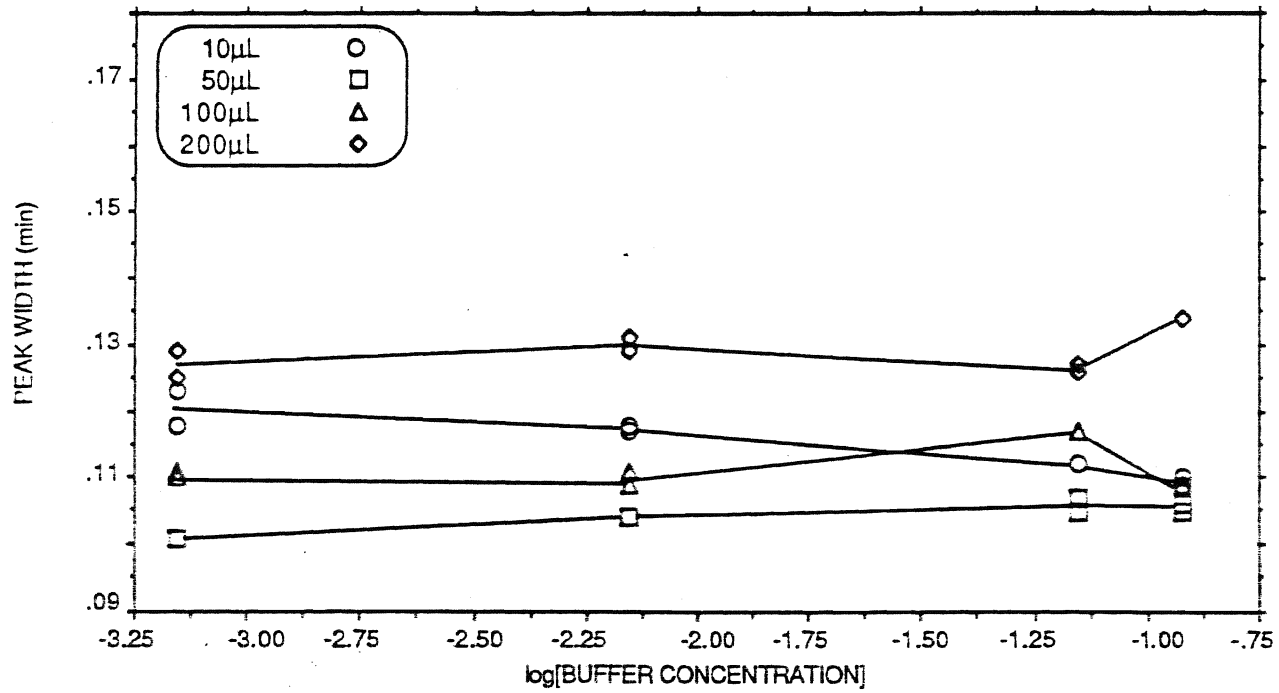
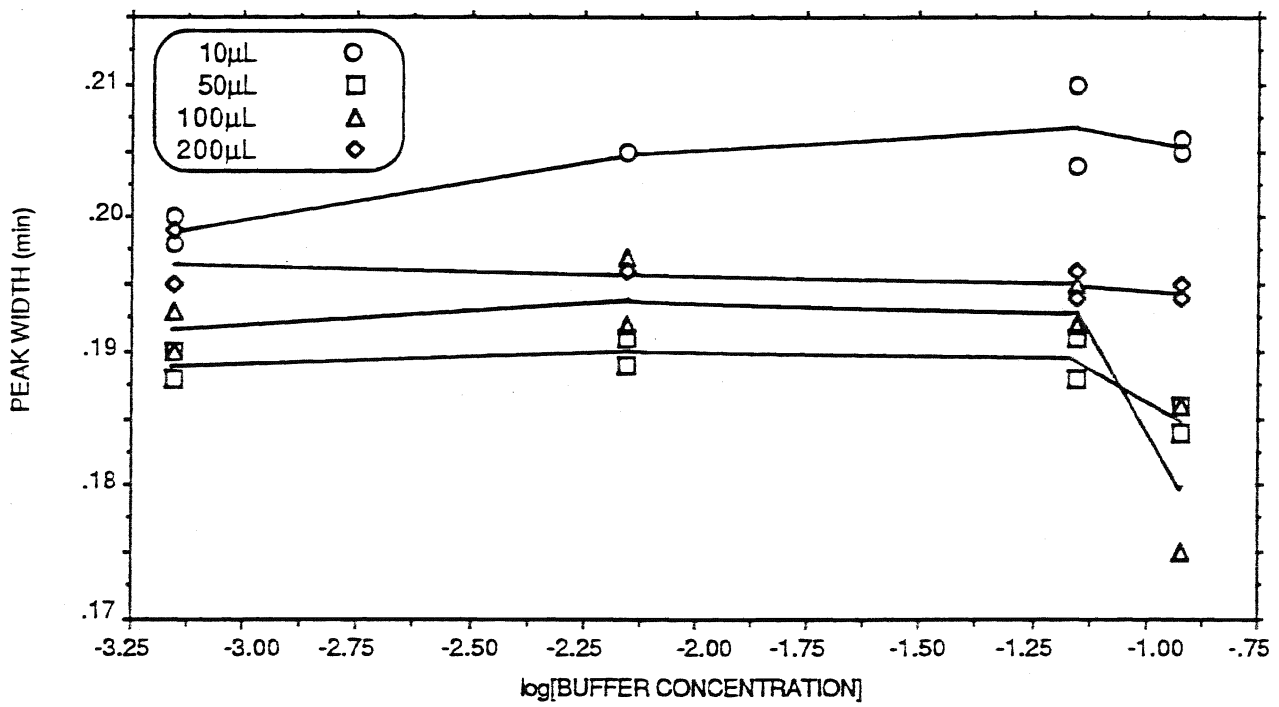


Figure 54: Plot of Peak Width vs. log[Buffer Concentration] for MBC at Constant Analyte Mass for 10, 50, 100, and 200 µL Injections.



for 10, 50, and 100 µL injections. The increases became smaller as the injection volume became larger. For 200 µL injection there was no observable effect (Table XVIIIa and Figure 49). This trend was very similar to the methanol study at constant mass. The decreasing influence of buffer concentration on the retention time of MBC was observed only at 0.12 M concentration for 50 µL injection, and for 0.07 and 0.12 M concentrations at 100 and 200 µL injections (Table XVIIIb and Figure 50).

Peak Height An increase in the STB peak height of 19.7% was observed only for 10 µL injections, where analyte concentrations were 5 µg/mL. For 50 and 100 µL injections, where analyte concentrations were 5 and 10 times lower, a very gradual decrease in the peak height was observed. For 200 µL injection, where analyte concentrations were 20 times smaller no obvious trend was observed (Figure 51). The buffer concentration had no significant effect on the peak heights of MBC at any of the four volumes studied, as shown in Figure 52.

Peak Width Peak widths for STB decreased at 10 µL injection and showed no pronounced change 50, 100, and 200 µL injections (Figure 53). Peak widths of MBC were not influenced at any volume by the increase in the buffer strenght (Figure 54). For both analytes somewhat irregular behavior was observed for the highest buffer concentration of 0.12M.

Peak Area The peak area of STB was not greatly influenced by the buffer concentration (Table XVIIIa). A small reduction in

the peak area of STB was observed only at 5 µg/mL for 10 µL injection when no buffer was present in the sample solution, or at very low buffer concentration of 0.0007 M. For MBC the only effect was a loss of reproducibility at highest buffer concentration and highest injection volume.

Symmetry For 10 µL injection, symmetry was constant for STB. For 50, 100, and 200 µL injections, a small increase in the symmetry was observed. Peak symmetry of MBC did not change.

2) At Increasing Mass of Analyte Injected

The complete results are listed in Tables XIXa and XIXb and the sample chromatograms are shown in Figure 55.

Retention Time An increase in the STB retention time of 2.5 and 3.0% were observed for 10 and 50 µL injection volumes. The only other change was a decrease in retention time for buffer concentration increase from 0.07 to 0.12M at 200 µL injection as shown in Figure 56. For MBC, the only influence was a small decrease at 100 and 200 µL injections of 1.3 and 1.0% as observed in Figure 57.

Peak Height STB peak heights increased at higher buffer concentrations as shown in Figure 58. The increases were 19.7, 9.5, 18.9 and 8.2% for 10, 50, 100, and 200 µL injections respectively. An interesting factor here was the variation in the increases at different injection volumes. This will be discussed in section IV. Peak height of MBC was not affected except for a small increase at the highest buffer concentration as shown in Figure 59. This was also the case in

Table XIXa

Chromatographic Results for STB when [Buffer] in the Sample Solvent increases from 0 to 0.12M and Injected Mass of Analytes Increases with Increasing Injection Volume.

[BUFFER] M	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10µL @ 5µg/mL			
0.00	2.247±.009	80 ±1	9.35 ±.14	0.121±.001	0.37 ±.01
0.00	2.250±.004	84 ±6	9.57 ±.14	0.122±.006	0.35 ±.02
0.0007	2.254±.002	82 ±1	9.50 ±.03	0.118±.002	0.36 ±.02
0.0007	2.254±.003	82 ±4	9.18 ±.06	0.123±.004	0.35 ±.01
0.007	2.260±.004	85 ±1	10.13 ±.13	0.117±.001	0.34 ±.02
0.007	2.263±.001	86 ±2	10.08 ±.02	0.118±.003	0.36 ±.01
0.07	2.293±.002	89 ±2	11.15 ±.25	0.112±.001	0.35 ±.02
0.07	2.288±.004	84 ±1	11.07 ±.29	0.112±.003	0.37 ±.02
0.12	2.306±.003	89 ±4	11.30 ±.33	0.110± 0	0.37 ±.02
0.12	2.306±.005	88 ±1	11.34 ±.17	0.109±.002	0.37 ±.02
		50 µL @ 5µg/mL			
0.00	2.377± 0	86 ±1	10.60±.57	0.119±.008	0.38 ±.03
0.00	2.374±.004	87 ±1	10.60±.45	0.119±.009	0.39 ±.03
0.0007	2.374±.001	87 ±1	10.57±.57	0.120±.007	0.40 ±.01
0.0007	2.378±.001	84 ±1	10.26±.56	0.119±.009	0.40 ±.01
0.007	2.376±.001	90 ±1	11.23±.53	0.115±.005	0.39 ±.01
0.007	2.382 ±.011	90 ±1	11.10±.42	0.116±.005	0.39 ±.02
0.07	2.415±.004	93 ±1	11.36±.54	0.114±.006	0.34 ±.01
0.07	2.408±.007	93 ±1	11.41±.74	0.114±.007	0.34 ±.01
0.12	2.451±.001	93 ±1	11.60±.86	0.113±.009	0.33 ±.01
0.12	2.448±.033	94 ±1	11.61±.87	0.113±.008	0.33 ±.02
		100 µL @ 5µg/mL			
0.00	2.483 ±.039	86 ±2	9.89± .45	0.127±.007	0.44 ±.07
0.00	2.484 ±.041	87 ±2	9.80± .55	0.128±.008	0.44 ±.06
0.0007	2.489 ±.045	86 ±1	9.89± .65	0.125±.007	0.44 ±.06
0.0007	2.483 ±.033	83 ±1	9.50± .47	0.125±.005	0.43 ±.05
0.007	2.473 ±.021	91 ±1	10.49± .41	0.124±.005	0.38 ±.02
0.007	2.466 ±.010	89 ±2	10.42± .41	0.123±.007	0.38 ±.04
0.07	2.506 ±.023	95 ±1	11.10± .91	0.120±.011	0.32 ±.02
0.07	2.497 ±.020	95 ±1	11.06± .88	0.120±.011	0.32 ±.02
0.12	2.493 ±.006	95 ±0	11.71 ±1.08	0.113±.012	0.31 ±.01
0.12	2.492 ±0	95 ±1	11.72 ±1.07	0.113±.012	0.31 ±.01
		200µL @ 5µg/mL			
0.00	2.589±.014	86 ±1	9.08 ±.29	0.137±.005	0.37 ±.06
0.00	2.588±.009	86 ±1	9.19 ±.26	0.135±.004	0.38 ±.04
0.0007	2.606±.020	85 ±1	9.23 ±.35	0.134±.004	0.37 ±.04
0.0007	2.589±.006	83 ±1	8.93 ±.29	0.134±.004	0.37 ±.04
0.007	2.587±.002	91 ±1	9.31 ±.31	0.142±.008	0.32 ±.02
0.007	2.586±.002	89 ±1	9.20 ±.40	0.140±.007	0.32 ±.02
0.07	2.587±.002	95 ±1	10.11 ±.09	0.133±.002	0.28 ±.01
0.07	2.581±.001	94 ±1	10.03 ±.02	0.134±.001	0.32 ±.06
0.12	2.554±.002	96 ±1	9.89 ±.06	0.139±.001	0.26 ±.01
0.12	2.554±.002	95 ±1	9.88 ±.22	0.138±.003	0.25 ±.01

TABLE XIXb

Chromatographic Results for MBC when [Buffer] in the Sample Solvent increases from 0 to 0.12M and Injected Mass of Analytes Increases with Increasing Injection Volume.

[BUFFER] M	RETENTION TIME (min)	AREA (mAUs)	PEAK HEIGHT (mAU)	PEAK WIDTH (min)	SYMMETRY
		10µL @ 5µg/mL			
0.00	3.312±.018	109±1	7.82±.16	0.203±.004	0.38±.02
0.00	3.318±.018	109±2	7.85±.14	0.198±.001	0.39±.02
0.0007	3.314± 0	111±1	8.01±.01	0.198±.003	0.39±.02
0.0007	3.315±.001	113±2	8.02±.09	0.200±.001	0.40±.01
0.007	3.322±.004	112±2	7.87±.07	0.205±.008	0.39±.01
0.007	3.323±.002	110±1	7.83±.08	0.205±.002	0.39±.01
0.07	3.323±.001	111±1	7.89±.04	0.204±.006	0.39±.01
0.07	3.318±.001	112±3	7.84±.08	0.210±.002	0.38±.01
0.12	3.320±.002	112±2	7.85±.05	0.206±.004	0.39±.01
0.12	3.322±.003	111±3	7.88±.17	0.205±.006	0.38±.02
		50 µL @ 5µg/mL			
0.00	3.370±.002	120±1	8.95±.02	0.196±.001	0.35±.01
0.00	3.368±.002	119±1	8.85±.01	0.197±.002	0.35±.01
0.0007	3.368±.005	121±3	8.86±.01	0.199±.004	0.34±.01
0.0007	3.370±.001	120±2	8.88±.03	0.199±.001	0.35±.01
0.007	3.367±.009	119±1	8.84±.01	0.197± 0	0.35±.01
0.007	3.376± 0	120±3	8.83±.03	0.198±.002	0.34±.01
0.07	3.365±.001	119±0	8.89±.05	0.197±.001	0.35±.01
0.07	3.366±.003	118±1	8.87±.05	0.196±.001	0.35±.01
0.12	3.373±.010	120±2	8.93±.01	0.195±.003	0.35±.01
0.12	3.384±.029	121±1	8.95±.04	0.197± 0	0.35±.01
		100µL @ 5µg/mL			
0.00	3.453±.031	122±2	9.12±.14	0.196±.006	0.36±.01
0.00	3.452±.036	122±1	9.05±.12	0.199±.004	0.35±.01
0.0007	3.457±.038	119±1	9.00±.07	0.195±.001	0.36±.01
0.0007	3.451±.024	120±1	9.02±.10	0.195±.003	0.35±.01
0.007	3.438±.014	122±1	9.06±.11	0.193±.007	0.36±.03
0.007	3.428±.006	121±3	9.04±.07	0.197±.004	0.35±.01
0.07	3.435±.018	122±0	9.20±.13	0.195±.003	0.35±.01
0.07	3.424±.012	123±0	9.17±.10	0.195±.003	0.34±.01
0.12	3.409±.001	123±1	9.32±.13	0.192±.005	0.35±.01
0.12	3.406±.007	124±1	9.41±.16	0.192±.002	0.35±.01
		200µL @ 5µg/mL			
0.00	3.534±.003	122±3	9.18±.18	0.196±.004	0.35±.01
0.00	3.531±.008	121±3	9.09±.14	0.197±.001	0.35±.01
0.0007	3.543±.020	121±2	9.06±.13	0.198±.001	0.34±.01
0.0007	3.533±.001	121±2	9.07±.08	0.197±.004	0.34±.01
0.007	3.530± 0	121±3	8.89±.16	0.203±.007	0.35±.01
0.007	3.529± 0	121±3	8.89±.16	0.208±.007	0.35±.01
0.07	3.516±.001	124±1	9.06±.18	0.203±.004	0.34±.01
0.07	3.512±.002	123±1	9.00±.14	0.204±.004	0.35±.01
0.12	3.497±.004	124±1	9.16±.13	0.204±.007	0.35±.02
0.12	3.494±.006	124±1	9.17±.04	0.200±.001	0.36±.01

Figure 55: Chromatograms of STB (1) and MBC (2) for Increasing Mass Study Showing the Effects of Increasing Buffer Concentration in the Sample Solvent: 1) 0.0M; 2) 0.0007M; 3) 0.007M; 4) 0.07M; 5) 0.12M.

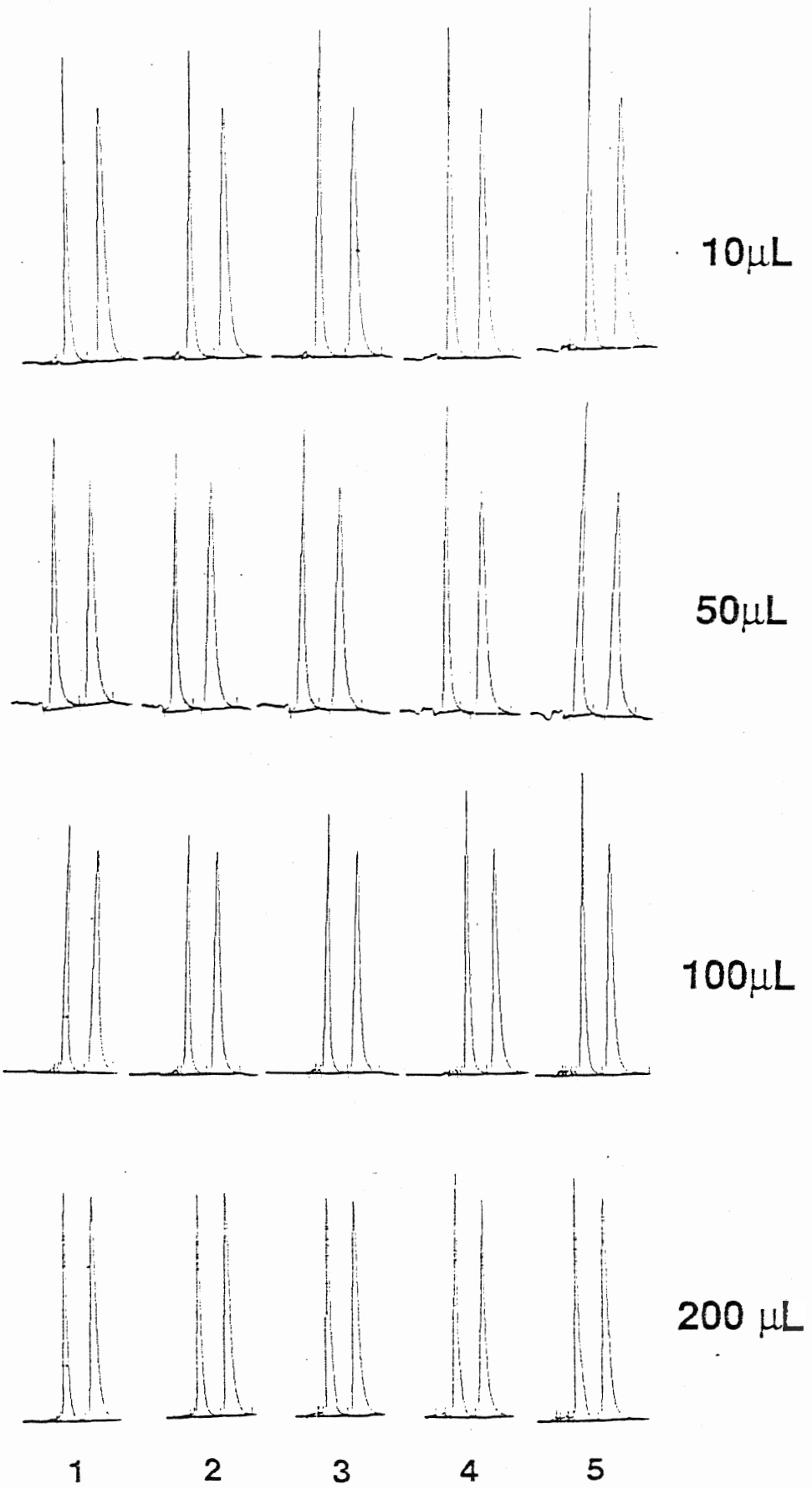


Figure 56: Plot of Retention Time vs. log[Buffer Concentration] for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

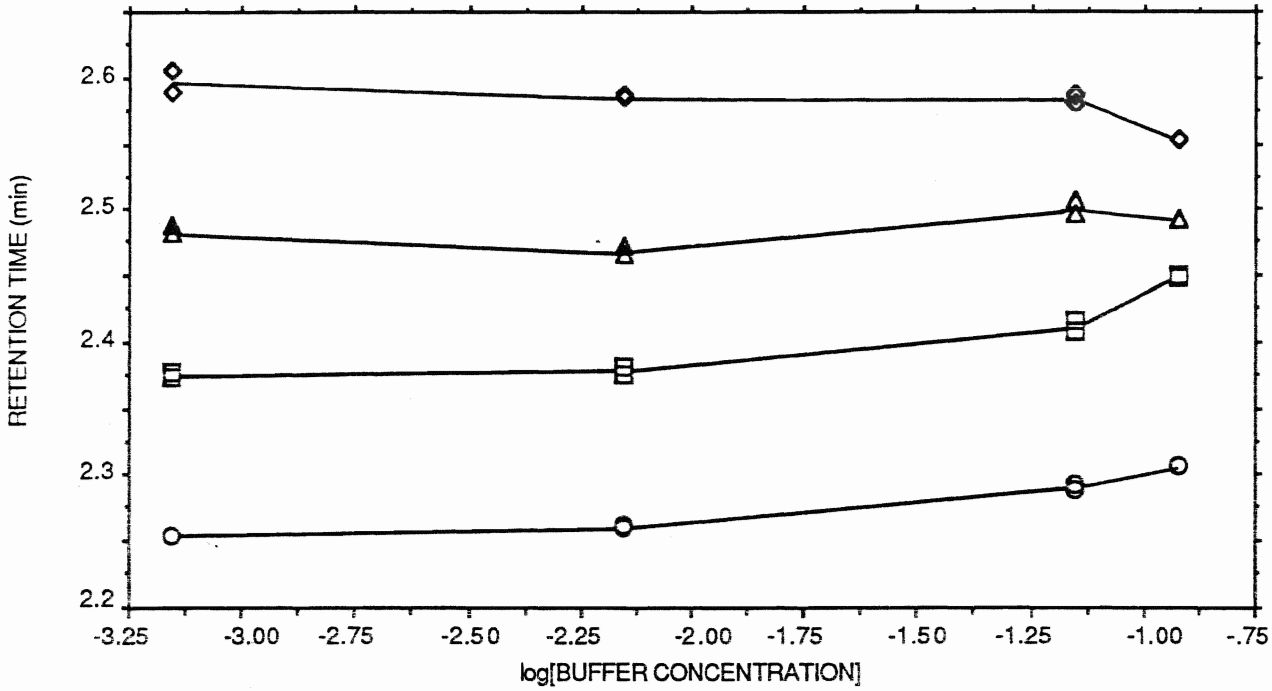


Figure 57: Plot of Retention Time vs. log[Buffer Concentration] for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

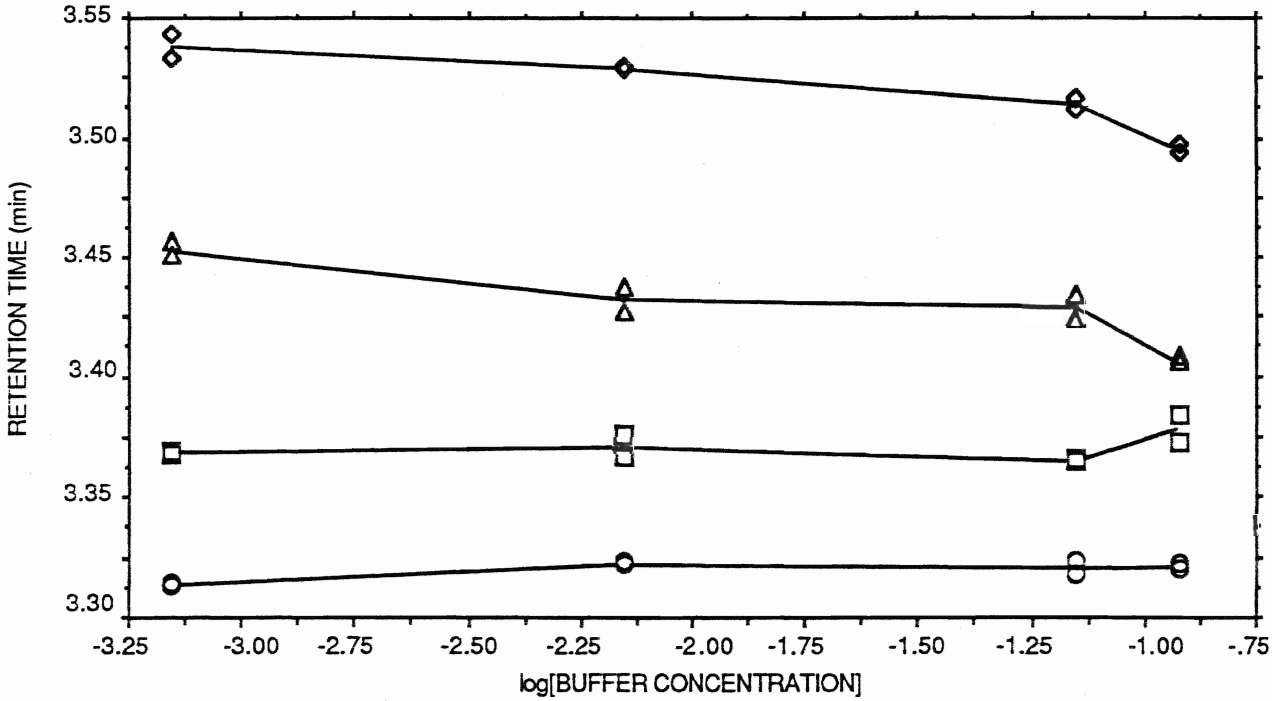


Figure 58: Plot of Peak Height vs. log[Buffer Concentration] for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

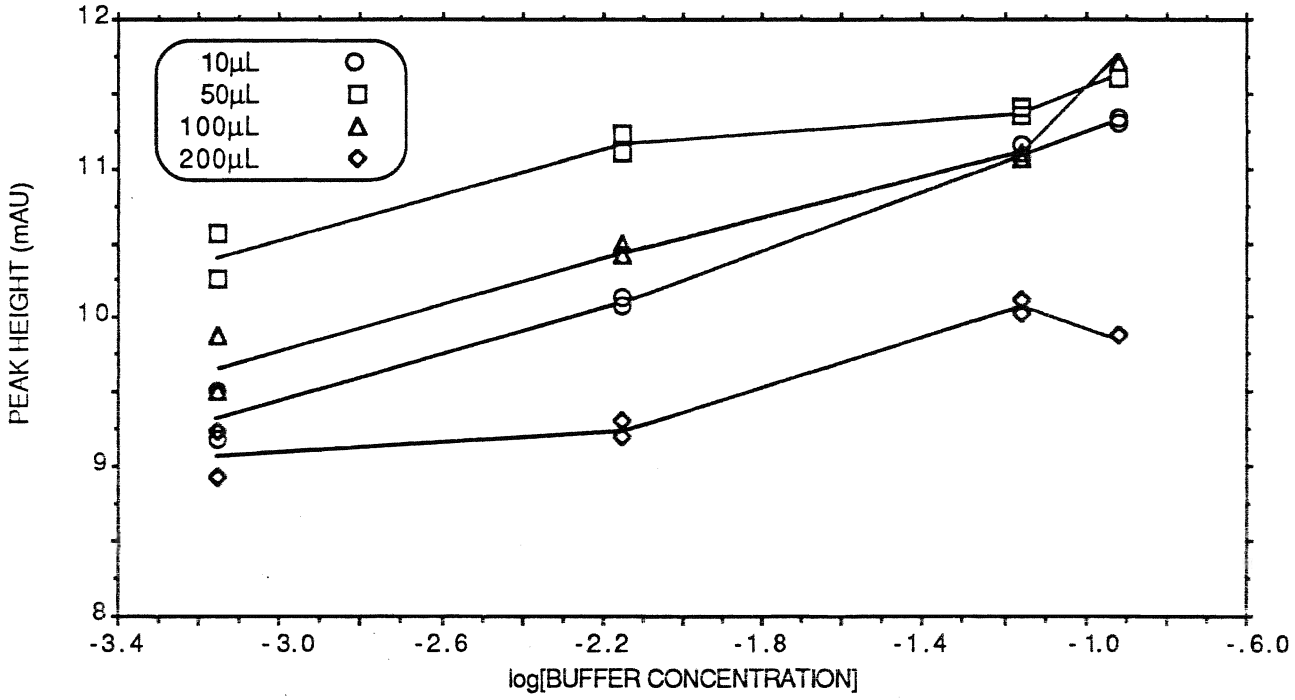


Figure 59: Plot of Peak Height vs. log[Buffer Concentration] for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.

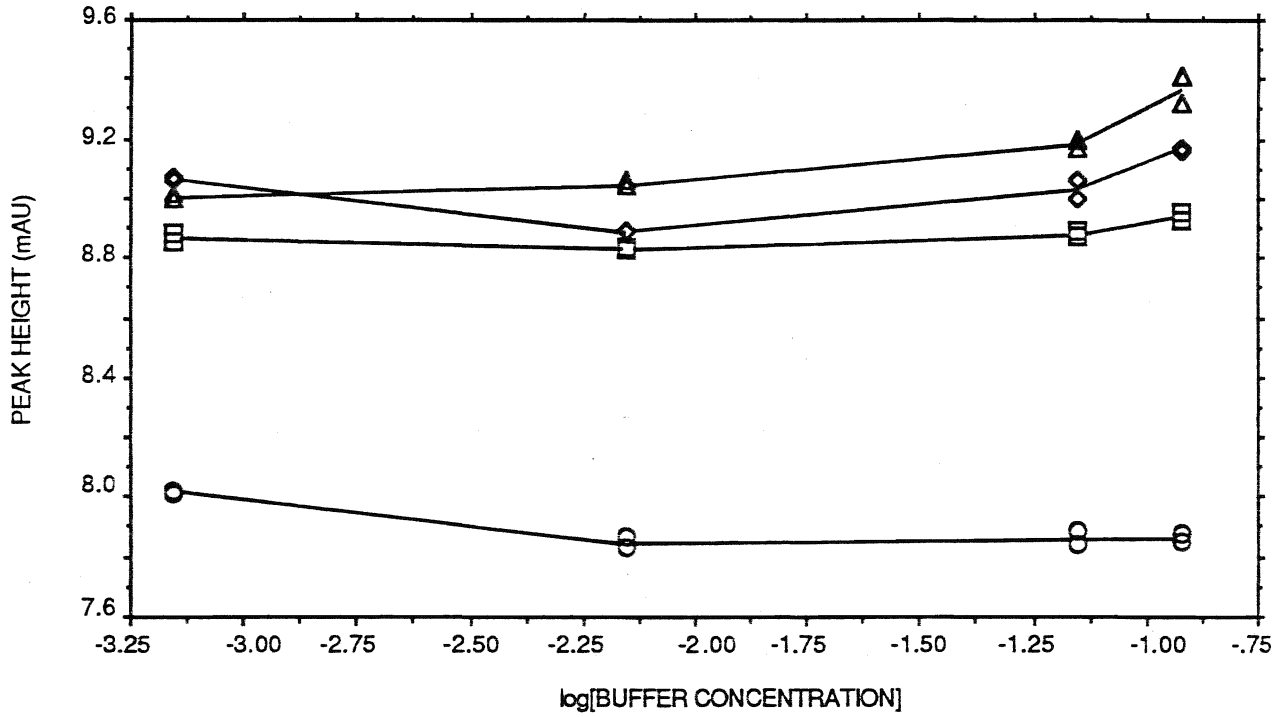


Figure 60: Plot of Peak Width vs. log[Buffer Concentration] for STB at Increasing Analyte Mass for 10, 50, 100 and 200 µL Injections.

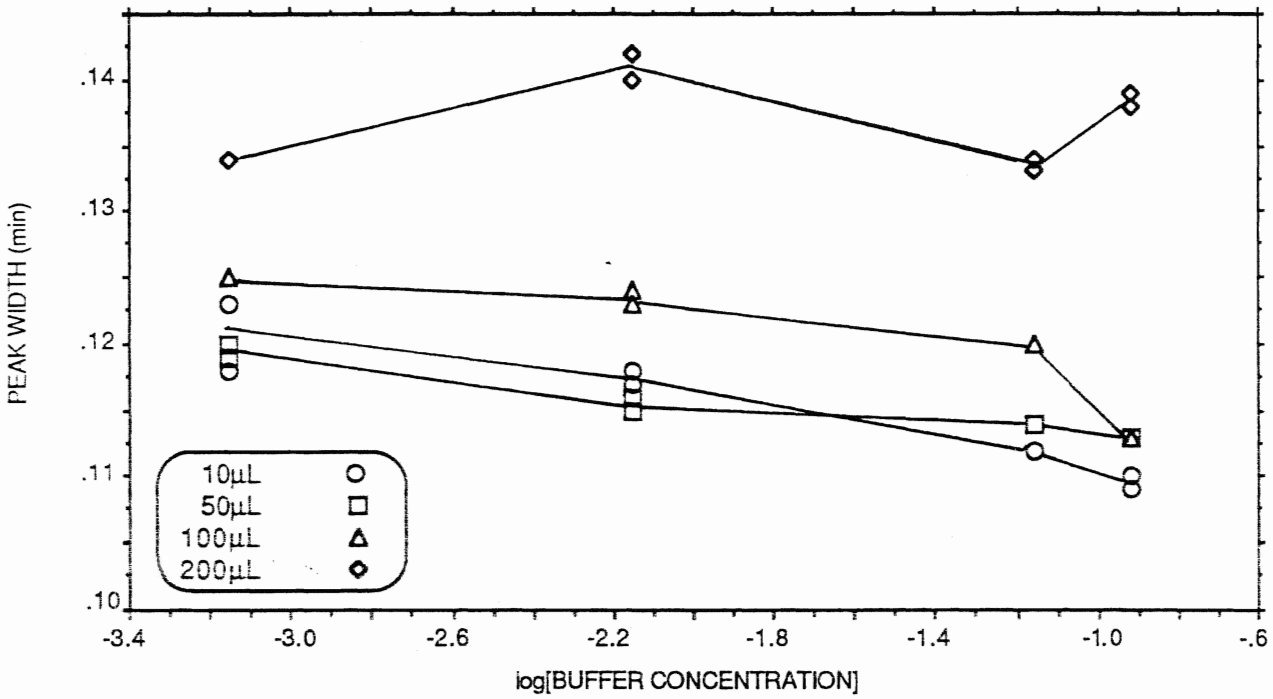
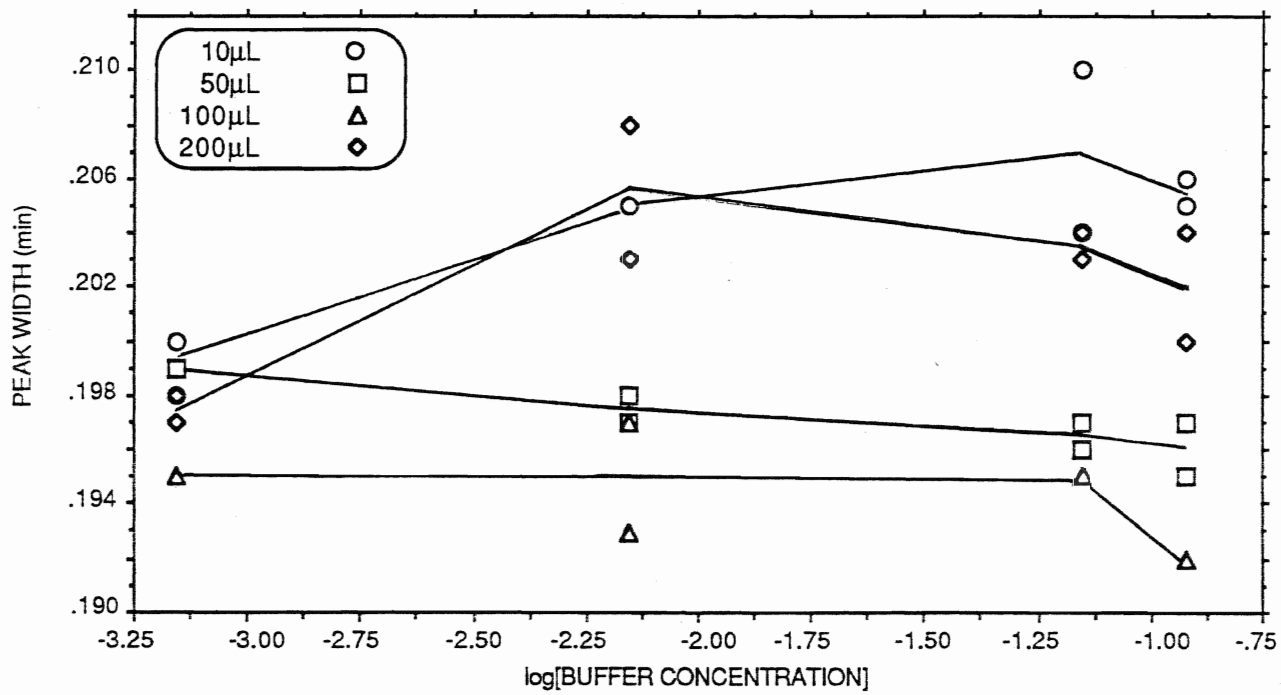


Figure 61: Plot of Peak Width vs. log[Buffer Concentration] for MBC at Increasing Analyte Mass for 10, 50, 100, and 200 µL Injections.



the constant mass study.

Peak Widths A small decrease in the STB peak widths was observed for 10, 50, and 100 µL injections at higher buffer concentrations. At 200 µL injection, no trend could be established. The results are shown in Figure 60. There was no pronounced influence on the MBC peak width, as can be seen in Figure 61.

Peak Area As the buffer concentration increased from 0 to 0.12 M, an increase in the peak area of STB was observed. The possible reasons for this will be discussed in section IV. The peak area of MBC was not affected by the change in the buffer strength.

Peak Symmetry For STB, small increases in the peak symmetry were observed for 50, 100, and 200 µL injections while MBC symmetry was not influenced by the buffer concentration.

E. Additional Experiments

1.Sample Solution pH

The true pH values in a buffered water-organic solvent medium could not be measured accurately with the conventional pH meter and the aqueous buffer standards due to the difference in the liquid junction potential and the activity coefficients in the aqueous and the mixed solvents (75). For this reason pH values of the buffer solutions which were added

Table XX

pH of Buffer Added and the Apparent pH* of the Sample Solutions at Various Compositions.

% CH_3CN in Sample Sol.	pH of Buffer Added	Apparent pH* of Sample Sol.
5.0	7.00±.02	7.36±.01
25.0	7.00±.02	7.74±.02
35.0	7.00±.02	7.92±.04
50.0	7.00±.02	8.20±.04

%CH_3OH in Sample Sol.	pH of Buffer Added	Apparent pH* of Sample Sol.
5.0	7.00±.02	7.38±.01
25.0	7.00±.02	7.83±.04
35.0	7.00±.02	8.12±.01
50.0	7.00±.02	8.61±.03

[Buffer] M	pH of Buffer Added	Apparent pH* of Sample Sol.
0.0000	7.00±.02	6.11±.05
0.0007	7.00±.02	7.36±.20
0.0070	7.00±.02	7.35±.01
0.0700	7.00±.02	7.35±.08
0.1200	7.00±.02	7.13±.01

Buffer pH	pH of Buffer Added	Apparent pH* of Sample Sol.
5.0	5.00±.02	5.59±.01
6.0	6.00±.02	6.35±.03
7.0	7.00±.02	7.38±.01
8.0	8.00±.02	8.22±.07
Mobile Phase	7.00±.02	7.97±.10

to the sample solution, and the apparent pH* of the solution are reported in Table XX. A significant influence of the organic solvent concentration in the sample solvent on the apparent pH* was observed. As the concentration of the acetonitrile increased from 5 to 50%, the apparent pH* increased from 7.36 to 8.20. The increase in the methanol concentration from 5 to 50% produced an increase in the apparent pH* from 7.38 to 8.61. The apparent pH* of the mobile phase, which contained 35% acetonitrile, was 7.97. This pronounced influence of the organic solvents on the apparent pH* of the phosphate buffered sample solutions can be attributed to the influence of the organic solvents on the pKa values of the buffered salts. The similar influence on the phosphate buffers was reported by Leitold and Vigh (57).

The increase in the buffer concentration and therefore in the ionic strength of the sample solution, from 0.0007 to 0.07, gave constant apparent pH* readings of 7.3. For the solution with buffer concentration of 0.12 M, apparent pH* was 7.13.

When the pH of the added buffer was varied, the observed pH* of the sample solution was influenced to different extent as can be seen in Table XX. This difference in the apparent pH* can be attributed to the variation in the buffering capacity of the phosphate buffer.

2.Ultra Violet Spectra of Sample Solutions

The UV absorbance profiles of the two compounds at various sample solvent compositions, presented some interesting observations as shown in Figure 62a. The absorbance profiles for MBC appeared unaffected by the changes in the organic solvent concentrations, in the ionic strength or in the pH of the sample solutions. The possible reasons will be discussed in the section IVA. For STB the absorbance profiles were affected quite markedly by the changes in the sample solvent composition. The most pronounced changes occured in the pH study as shown in Figure 62a. The changes in the absorbance profiles for the remaining three studies also seem to be dependent on the pH of the solution. For the buffer concentration sudy the absorbance profile for the unbuffered soultion at pH of 6.11 was quite different from the buffered solutions. It was however quite similar to the absorbance profile for the solution at pH 6.35 in the pH study. The profiles for the solutions at varying ionic strength but the same pH were very similar. For 0.12 M buffer concentration, where pH was slightly lower, absorbance maximum was shifted back to slightly higher wavelength. The changes in the absorbance profiles for the solutions at increasing organic solvent concentrations seem to be related to the increase in the apparent pH*. The increase in the organic solvent resulted in the broader absorption bands with the absoption maxima

Table XXI

Influence of Sample Solvent Composition on the UV Absorbance Profile of STB and MBC

% CH_3CN in Sample Sol.	λ_{max} MBC	λ_{max} STB	Abs. at λ_{max} MBC	Abs. at λ_{max} STB	Abs. at λ_{280nm} MBC	Abs. at λ_{280nm} STB
5.0	279.3	279.1	0.377	0.265	0.382	0.269
25.0	279.8	276.4	0.370	0.273	0.375	0.273
35.0	279.5	276.2	0.365	0.277	0.371	0.274
50.0	280.0	274.9	0.359	0.280	0.364	0.273

%CH_3OH in Sample Sol.	λ_{max} MBC	λ_{max} STB	Abs. at λ_{max} MBC	Abs. at λ_{max} STB	Abs. at λ_{280nm} MBC	Abs. at λ_{280nm} STB
5.0	279.3	279.5	0.379	0.264	0.385	0.269
25.0	279.3	276.2	0.380	0.275	0.382	0.275
35.0	279.3	275.5	0.376	0.282	0.381	0.278
50.0	279.3	275.5	0.374	0.294	0.380	0.284

[Buffer] M	λ_{max} MBC	λ_{max} STB	Abs. at λ_{max} MBC	Abs. at λ_{max} STB	Abs. at λ_{280nm} MBC	Abs. at λ_{280nm} STB
0.0000	279.6	282.5	0.367	0.269	0.367	0.263
0.0007	279.6	278.5	0.367	0.269	0.367	0.261
0.0070	279.6	278.1	0.366	0.269	0.368	0.263
0.0700	279.6	278.5	0.368	0.264	0.370	0.265
0.1200	279.4	278.5	0.371	0.265	0.374	0.268

Buffer pH	λ_{max} MBC	λ_{max} STB	Abs. at λ_{max} MBC	Abs. at λ_{max} STB	Abs. at λ_{280nm} MBC	Abs. at λ_{280nm} STB
5.0	280.7	282.9	0.389	0.274	0.385	0.272
6.0	280.7	282.9	0.383	0.271	0.383	0.272
7.0	279.5	278.9	0.383	0.266	0.380	0.270
8.0	280.3	273.1	0.380	0.283	0.383	0.270

Figure 62a: Effects of Sample Solvent Composition on the UV Absorbance Profiles of STB and MBC from 340 to 240 nm before Chromatographic Analysis: 1) Acetonitrile Increase; 5 to 50%; 2) Methanol Increase; 5 to 50%; 3) pH Increase; 5.0 to 8.0; 4) Buffer Concentration Increase; 0.00 to 0.12M.

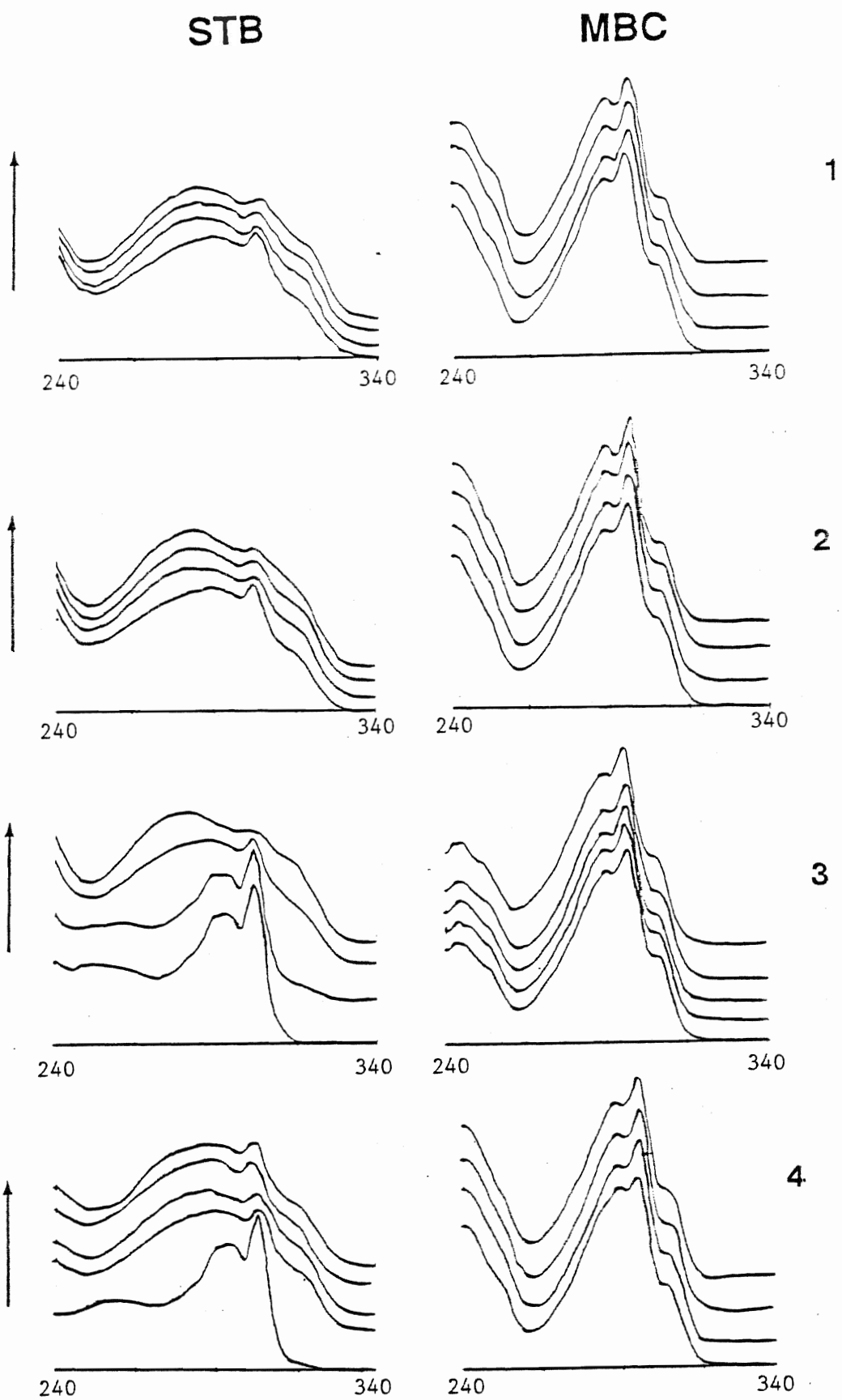
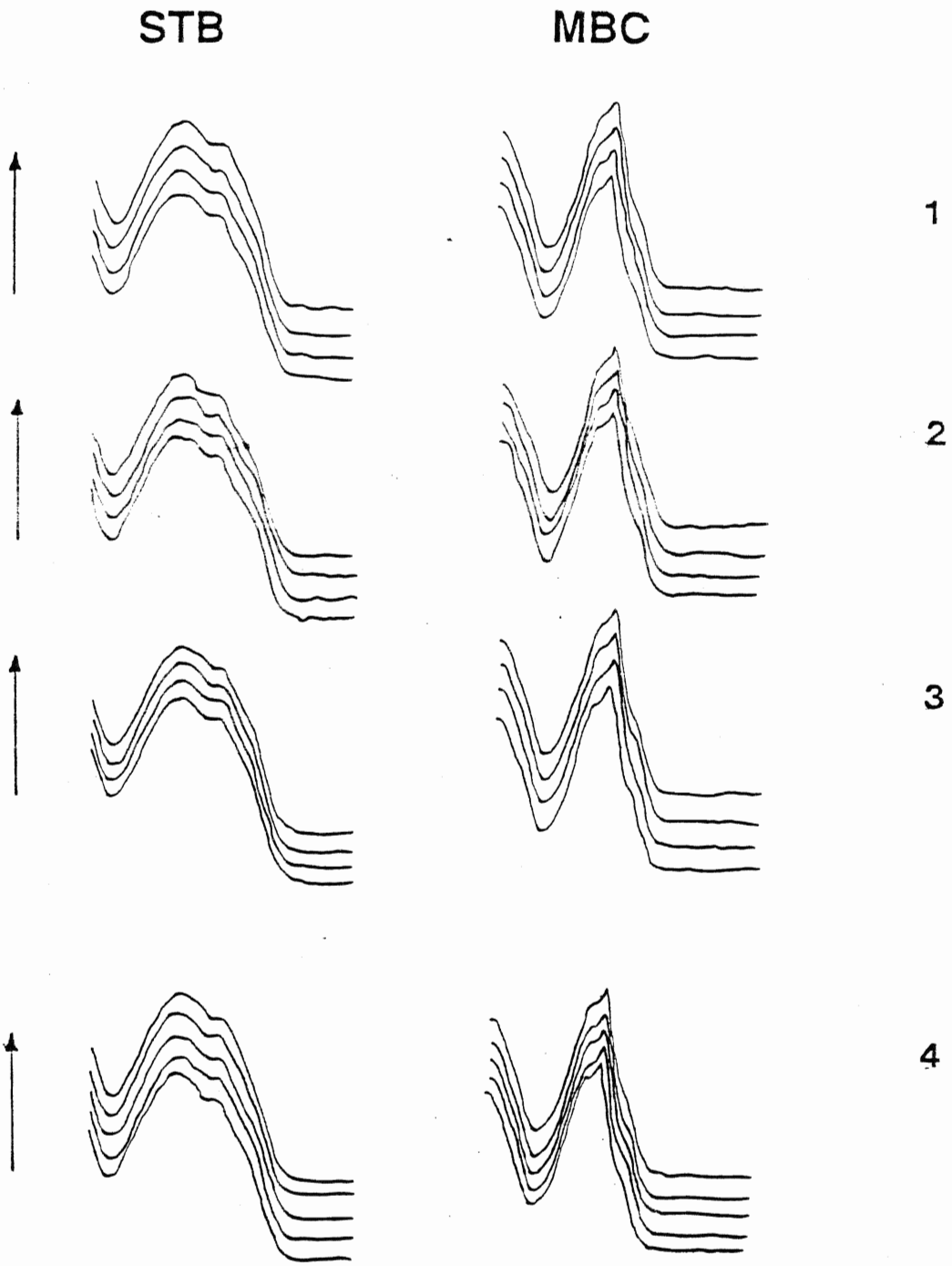


Figure 62b: UV Absorbance Profiles of STB and MBC from 340 to 240 nm as they were Eluted from the HPLC Column:1) Acetonitrile Increase; 5 to 50%; 2) Methanol Increase; 5 to 50%; 3) pH Increase; 5.0 to 8.0; 4) Buffer Concentration Increase; 0.00 to 0.12M.



being shifted to lower wavelengths. This was similar to the trend observed at increasing pH in the pH study.

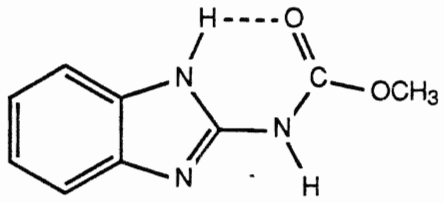
IV. Discussion

A.Compounds Studied

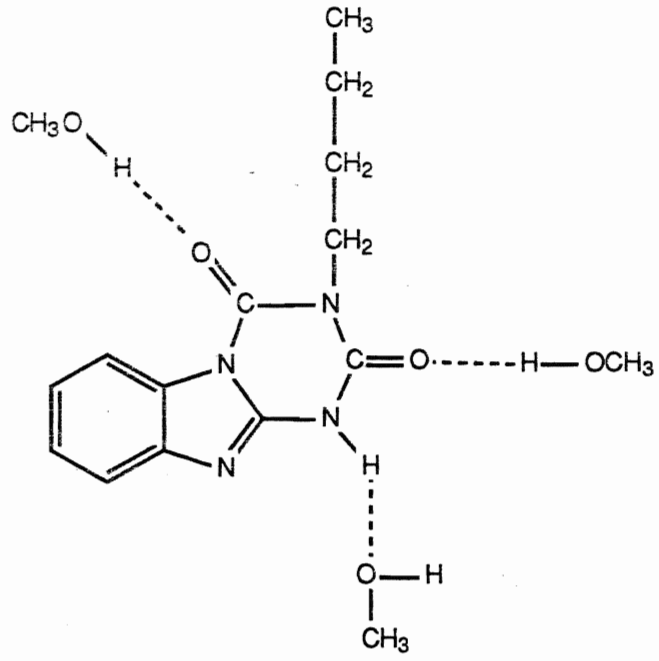
As mentioned in the introduction, the two compounds studied were degradation products of fungicide benomyl and were shown to possess fungitoxic properties themselves (79). They have in common a large benzimidazole moiety and an amide functionality and would be expected to have similar chromatographic properties. This, however, is not necessarily the case. During our study it became apparent that the two compounds can be influenced in some cases in very different ways by the sample solvent. The difference between the two compounds was also indicated earlier by Singh and Chiba (79). It was not completely understood why the two compounds exhibit different chromatographic behaviour. It was of interest to explain this different behavior, especially since there are some questionable explanations in the literature (70,82) for similar type of behavior. To achieve this we used several analytical methods to probe the behavior of the two compounds in the solution and to learn more about their chemical properties.

The molecular structure of MBC consists of the benzimidazole, amide and methoxy functionalities as shown in Figure 5 (II). It is smaller in size than STB and is capable of forming intramolecular hydrogen bonds (Figure 63). The absence of any solvent influence on the UV absorbance profiles

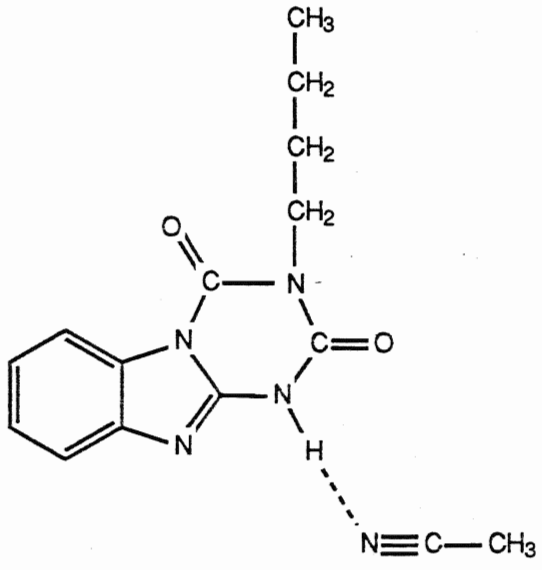
FIGURE 63: Hydrogen Bonding and Solute-Solvent Interactions for MBC and STB



1



2



3

of MBC (Figure 62a) suggests the following two possibilities. Firstly, the absence of organic solvent influence on the absorbance profile strongly suggests that the amide carbonyl oxygen was involved in the intramolecular hydrogen bonding as shown in Figure 63 and therefore unavailabe for solvent interaction. The carbonyl oxygen that is not involved in the hydrogen bonding was shown to be strongly influenced by the solvent polarity (83). Secondly, the absence of any pH influence suggests that amide and amine protons were not prone to the dissociation in the pH range tested. The involvement of a carbonyl oxygen in the intramolecular hydrogen bonding would minimize electron withdrawing presence from the amide nitrogen, making it more basic with higher dissociation constant and therefore much less influenced by the pH variations than STB. The intramolecular hydrogen bond formation would also decrease the tendency of the amide hydrogen to interact with the solvent molecules (84). The intramolecular hydrogen bond formation is also consistent with the absence of dimers in the FAB mass spectral analysis since carbonyl oxygen would not be available for dimer formation.

STB is a larger molecule consisting of a more rigid three ring structure and a freely moving butyl group which could have a major influence in the hydrophobic retention mechanism (Figure 5 III). It has no capability of forming intramolecular hydrogen bonds but it is capable of forming intermolecular bonds at three different positions as shown in Figure 63.

Table XXIIa

Retention Factor k'
for STB at Increasing pH

pH	k'
4.42	0.83
5.95	0.72
6.50	0.54
6.74	0.51
7.40	0.48
7.90	0.46
9.10	0.45

Table XXIIb

UV Absorbance Values at 291 nm
for STB at Increasing pH

pH	A(a.u.)
5.59	0.366
6.35	0.345
7.38	0.282
8.22	0.258

Figure 64: Plot of UV Absorbance vs. pH of the Sample Solution for STB at 291 nm.

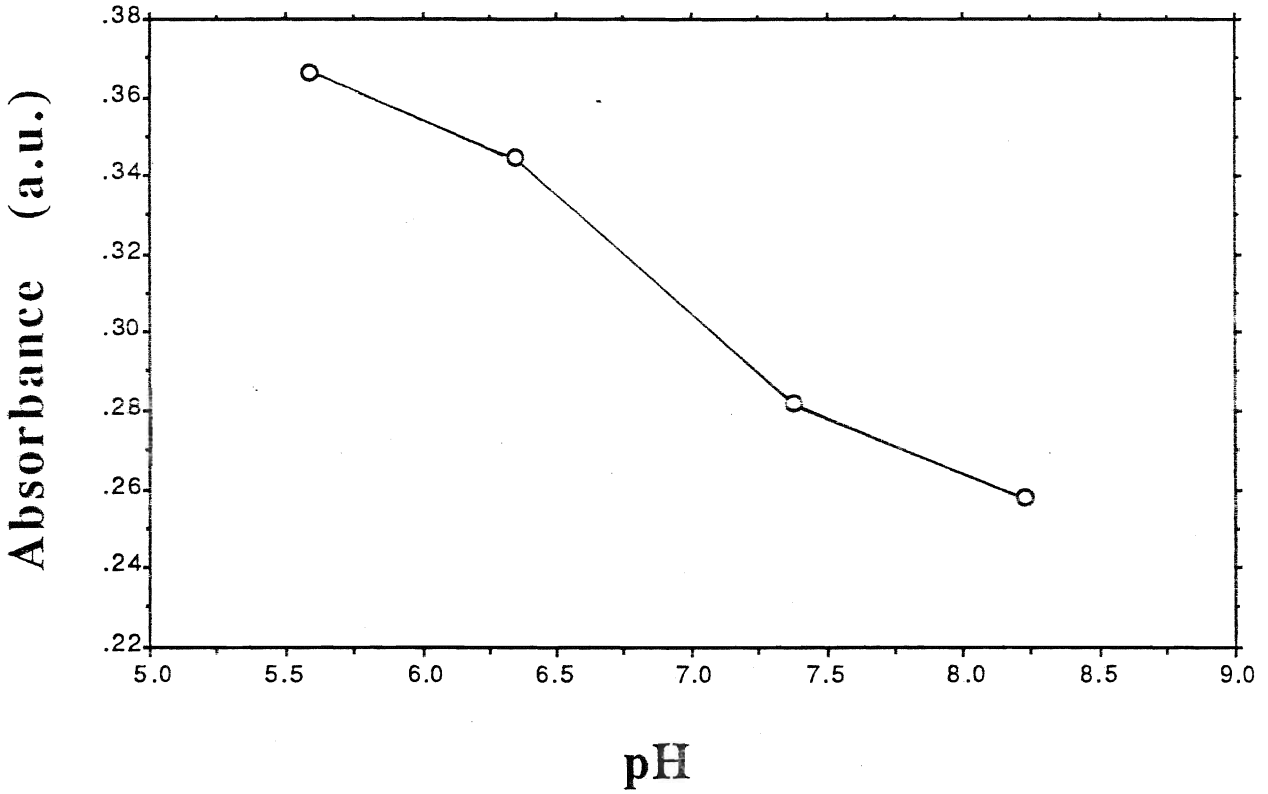
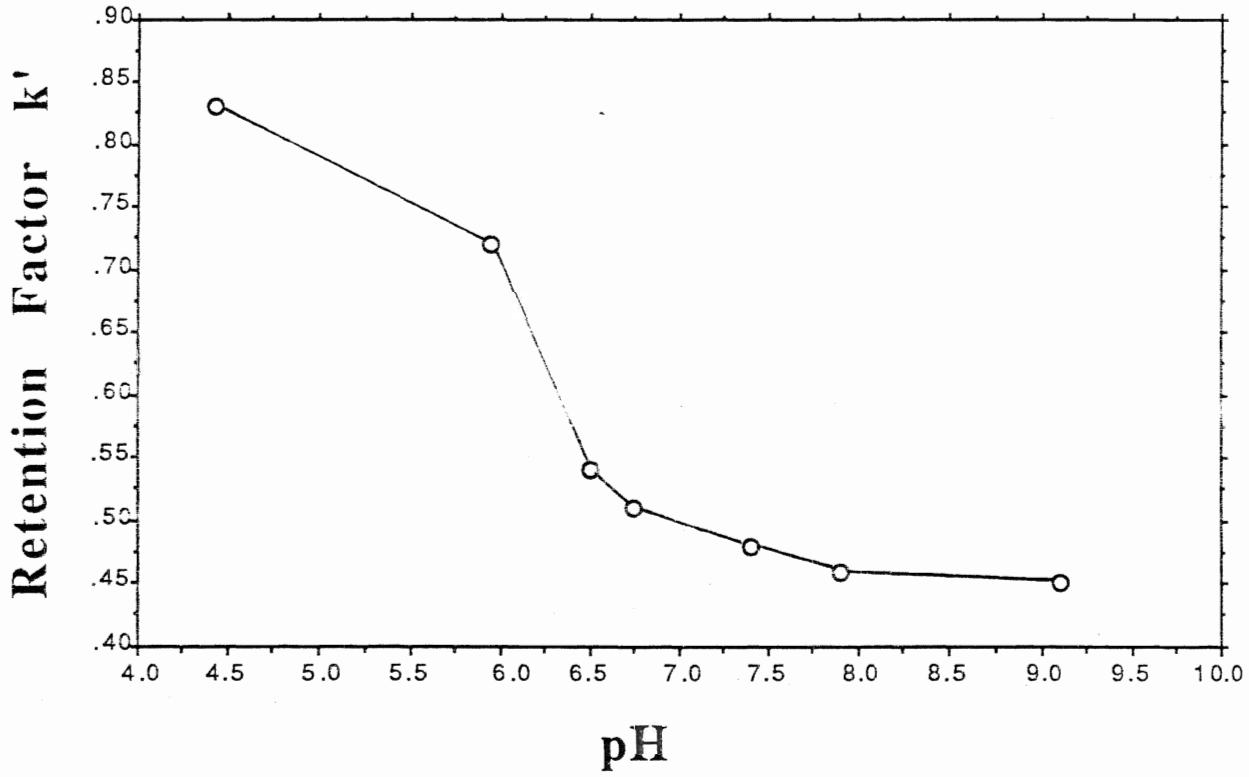


Figure 65: Plot of Retention Factor k' vs. pH of the Sample Solution for STB.



As demonstrated in Figure 62a, a significant influence of the sample solvent on the STB absorbance profile was observed. There are three possible explanations for these interactions. As reported in the results section, the most pronounced change occurred in the pH study and especially between pH 6 and pH 7. The changes in the other three factors could also be pH dependent since the pH* of the solutions increased as the concentration of the organic solvent in the solution increased. The pH influence suggests the possible occurrence of dissociation in this pH range. Jaffe and Orchin (83) reported that the dissociation constant Ka can be obtained by determining the absorption spectra of a series of solutions at constant concentrations but at varying apparent pH*, and by plotting absorbance vs pH* at constant wavelength. The middle of the inflection point can be estimated as the apparent pKa* of the analyte in the mixed solvent. The apparent pKa* for STB at 291 nm was estimated at 6.5 from the plot in Figure 64. The pKa for STB was also determined from the plot of retention factor k' vs pH of the sample solution (Figure 65) and was found to be in the 6.5 pH range. A second explanation for the change in the absorbance is the formation of the complex with the buffer species. At increasing pH, the amide proton becomes more acidic and therefore more prone to the interaction with the negatively charged buffer species, which would result in the band broadening. The third possibility could be the involvement of carbonyl oxygens in the hydrogen bond formation with protonated solvents such as methanol (Figure 63). It has

been shown that the absorption curves of diketones are influenced by the solvent and that the maxima are shifted to the shorter wavelength as the polarity of the solvent decreases (85). The formation of the intermolecular hydrogen bonding is also supported by the presence of STB dimers in the FAB mass spectral analysis in 3-nitrobenzyl alcohol. The significantly higher base peak for STB than MBC in the FAB analyses suggests much higher tendency of STB to ionize.

The UV absorbance profiles of both compounds, taken as they were eluted from the column after chromatographic separations, were the same in all the cases regardless of the sample solvent compositions and were identical to the absorbance profiles of the two compounds which were specifically prepared in the mobile phase solvents for the comparison purpose (Figures 62a and 62b). This result suggests that the analytes eluted from the column were determined by the detector without any influence from the sample solvent composition.

B. Effect of Sample Composition

The effect of the sample solvent composition on the chromatographic peak profile in RP-HPLC has been studied by several workers (63-77, 82), but most of these studies have been published in the last few years. This indicates that this topic is relatively new and gaining in importance.

At present the recommended procedure for RP-HPLC analyses is to inject the smallest volume possible (1-10 µL), and to dissolve the sample in the mobile phase in order to minimize baseline disturbances. In many instances adherence to this rule is not practical or is just not followed. Carbas et al. (80) analysed MBC and related fungicides using acetonitrile as the injection solvent and water:acetonitrile:phosphate buffer pH 7 (45:45:10) v/v % as the mobile phase solvent. They obtained very poor peak resolution and chromatographic efficiency. This was primarily due to the use of a sample solvent which is much stronger in eluting power than the mobile phase. In many cases mixtures of compounds to be analysed have a wide range of solubilities. For this reason two or more solvents are required to completely dissolve all the components. Because of this reason Chiba and Singh (65) used methanol, acetonitrile, and phosphate buffer in the sample solvent composition. They observed that the peak response and the resolution of STB and MBC were influenced by the pH, buffer concentration and the organic solvent concentrations.

The main objective of this research was to determine to what extent the peak profile is affected by changing the sample solvent compositions, to try to explain possible reasons for this influence, and to suggest possible ways to optimize sample compositions. For this purpose the effect of two common organic solvents (methanol and acetonitrile), and the effects of the solution pH and the buffer concentration

were examined.

1. Effects of Acetonitrile in the Sample Solution

Acetonitrile is one of the most important organic solvents in RP-HPLC. It is a relatively polar, nonprotic solvent which eluting strength in RP-HPLC (3.1) is slightly higher than that of methanol (3.0) and much higher than that of water (0) (48). It was observed that the acetonitrile concentration in the sample solvent showed a strong influence on the retention time, peak height and peak width for the both compounds studied (Figures 6-19). An increase in the acetonitrile concentration in the sample solution from 5 to 50% resulted in a significant decrease in the retention times and peak heights and an increase in the peak widths (Figure 7-12). The effects became more pronounced as the eluting strength of the sample solvent became higher than that of the mobile phase. The reason for the peak height reduction and peak broadening with an increase in the acetonitrile concentration can be attributed to the increase in the eluting strength of the sample solution. Ng and Ng (69) suggested that the effects of the sample solvent are also due to the change in the retention capacity of the column which results from the adsorption of the injection solvent onto the column. The peak broadening, which occured with the increase in the acetonitrile concentration, was in contrast to the mobile

phase effect where increase in the eluting strength usually results in the sharper peaks. The study demonstrated that the variations in the acetonitrile concentrations of only 10 or 20% could introduce significant errors into the quantitative results if peak heights are used in calculations. It also showed that the use of mobile phase composition in the sample preparation does not necessarily lead to the most efficient separation. For this reason solvents used in sample preparation should be aqueous or contain a minimum percentage of organic solvent necessary for complete dissolution of analytes.

At high injection volumes (100, 200 µL) and high acetonitrile concentrations (50%), split peaks were observed for STB but not for the longer retained MBC. Split peaks most often result when more than one retention mechanism is present or when a solute is preferentially soluble in one of the sample solvents (73-76). If peak splitting occurs, erroneous interpretation of the results could be obtained from more than one peaks regardless whether the peak height or area is used for measurements, if one is not aware of the peak splitting possibility and assumes that the extra peaks are due to sample impurities. Another important observation that came out of this study was the fact that the peak heights of the two analytes were affected to a different extent by the variation in the sample solvent composition (Figures IX and X). This observation has important implications for quantitative analyses by peak heights even if an internal standard is used,

since peak height responses may not be the same for the analyte and the standard.

Results in this study are consistent with the observations of several other groups that studied different types of compounds. Tsimidou and Macrae (68) found in their study of triglycerides, that sample solvents higher in eluting strength than the mobile phase may cause band broadening and in some cases peak-splitting. Since they used organic solvents for the sample dissolution which were different from the mobile phase, they attributed peak splitting to the incompatibility of the two solvents. In this study, peak splitting occured when the sample solvent was the same as the mobile phase (acetonitrile), and also when it was different (methanol). Williams et al. (67), in their study of sample solvent effect on Aspirin and related analgesics reported similar effects for acetonitrile. Ng and Ng (69) found that injection of decamethrin, a synthetic pyrethroid insecticide, in a solvent of stronger eluting power than mobile phase can produce two well resolved peaks. When the same compound was injected in the mobile phase, a single peak was observed. Perlman and Kirschbaum (70) and Kirschbaum and Perlman (86) observed that in a single component solvent such as water, methanol, acetonitrile or ethanol, significant reductions in the peak heights occured as the injection solvent became less polar. Since they observed this phenomenon only for compounds that are capable of forming intramolecular hydrogen bonding, they concluded that this is a phenomenon particular only to

these type of molecules. The results of this study disprove their theory since the reduction in peak heights occured for a compound capable of forming hydrogen bonding (MBC) and one that is not (STB). Berridge (82) also disputed Kirschbaum's conclusions. He reported reduction in the peak heights for captopril, which is capable of intramolecular hydrogen bond formation, and for p-hydroxybenzoate which cannot form intramolecular hydrogen bonds.

2. Effect of Methanol Concentration in the Sample Solution

The methanol concentration effects for MBC were very similar to those of acetonitrile, but were quite different for STB. The retention times and peak heights of MBC decreased and peak widths increased with an increase in methanol concentration. The behaviour for MBC could be attributed primarily to the increase in the eluting strength of the sample solvent. The influence of methanol on the STB peak profile was quite different. Here retention times and peak heights increased and peak widths decreased with an increase in methanol concentrations. At higher analyte concentrations and higher injection volumes (100 and 200 µL) peak splitting was observed (Table XIIIa). There are several interesting observations in the peak splitting pattern. As mentioned earlier, peak splitting usually indicates the presence of more than one factor that can effect retention mechanism, and this

seemed to be the case in the present study. At 100 µL injections, split peaks were observed at 25% methanol in the injection solvent. The areas of the split peaks were equal. The retention time of one peak was the same as that of MBC in 5% methanol solution and for the second peak was sharply increased. At 35 and 50 % methanol, no splitting was observed but a large increase in the peak width and significant change in the peak symmetry was observed. At 200 µL, split peaks occured for 25, 35 and 50% methanol concentrations. As the methanol concentration increased marked changes in the areas of the split peaks were observed. At 25 and 35% methanol the peaks which eluted first had a larger area while at 50% methanol the area of the longer retained peak became much larger, and the retention of the smaller first peak became much shorter (Table XIIIa).

There could be several reasons for methanol effects on STB that are quite different from the acetonitrile effects. The major reason could be the tendency of STB to form intermolecular hydrogen bonds with methanol through carbonyl oxygens and in that way alter to some extent the primary hydrophobic mechanism. In the presence of intermolecular hydrogen bonding, the conformation of the STB molecule may be changed in such a way to make the butyl branch more important in the retention mechanism. Since this is a nonpolar group it will be retained more strongly by the nonpolar C-18 stationary phase, resulting in a somewhat longer retention time. The intermolecular bond formation also results in higher

density of the sample solution which may reduce the initial diffusion of the sample molecules and result in narrower peaks. A second reason could be the modification of the stationary phase surface by methanol molecules. It was reported in previous papers (37,87) that methanol will interact with free silanols on the surface of the reverse phase packing material and make the surface more hydrophobic. Since STB possesses a large nonpolar n-butyl group, it would be more strongly retained than the more polar MBC. Titova et al. (88) suggested that the acetonitrile molecules may be adsorbed more strongly onto the stationary hydrophobic surface than more polar methanol molecules. This modification of the stationary phase could also explain why a more pronounced decrease was observed in the retention time for acetonitrile study than for methanol study. As the injection volume and the methanol content increase, the modification of the stationary phase and the conformational influence could become more important which would explain the observed increase in the peak area of the longer retained split peak. The less pronounced decrease in the peak heights of MBC for methanol study than was seen in acetonitrile study may also be due to fewer interactions between the methoxy group on MBC and the surface silanols. Another reason could be that as the methanol content increases up to 40%, the viscosity of the sample solvent also increases as was demonstrated by Colin et al. (89). The viscosity increase reduces sample diffusion which can result in increased peak height and reduced peak width.

Since STB has smaller retention factor k', this increase in efficiency for STB becomes more pronounced than for the longer retained MBC. As methanol concentration increases over 40%, the viscosity of the sample solution begins to decrease which may once again encourage band spreading. Around the same time, the solvent strength becomes higher than that of mobile phase, this also contributes to band broadening.

Williams et al. (67) also observed peak height increases for Aspirin and related analgesics, up to around 35% methanol in the sample solvent. Their findings, which were obtained using an ODS II column, eliminate any doubts that our results may be limited to a particular column.

3. Effect of pH in the Sample Solution

Increase in the pH of sample solvents from pH 4 to pH 9 resulted in a substantial increase in the peak heights and a decrease in the retention time and peak widths for STB (Figures 34-36 & Tables XIV-XVI). The retention time decrease became much more pronounced at higher buffer concentrations in the sample solvent (Table XIV). For MBC study, variations in the sample solvent pH up to pH 9 had no observable effect on the peak retention or peak profile. Only at pH 9 and at higher buffer concentration of 0.06M was a small decrease in the peak width observed. This difference in the sample solvent effects on the two compounds can again be explained by the difference

in the chemical properties of the two molecules. As mentioned earlier, MBC is more stable at higher pH and unlike STB, has no tendency to ionize in the pH range that was studied. Its capability to form an intramolecular hydrogen bond makes it also less prone to interactions with the sample solvent. Both of these factors could be responsible for the absence of sample solvent influence. STB on the other hand is more prone to ionization and intermolecular bond formation as pH increases (Figures 64-67). The ionized form of STB becomes less retained by the nonpolar stationary surface resulting in a decrease in the retention time. The weaker interaction with the stationary phase also results in less peak broadening and therefore in a corresponding peak height increase.

It appears that the effects of the sample solvent pH and of the mobile phase pH on STB peak profiles were very similar. Carbas et al.(80) in his study of benomyl and its degradation products found that an increase in the pH of the mobile phase caused a reduction in the retention time and an increase in the sharpness of the STB peaks. Similar effects were observed for other ionizable substances. Foley and May (90), in their study of secondary chemical equilibria of chlorobenzoic acids in RP-HPLC, found that increase in the pH of the mobile phase will result in increased selectivity and peak heights of the analytes.

The above results lead to the conclusion that by controlling sample solvent pH one can control the efficiency and the resolution of ionizable solutes. This is illustrated

very well in Figure 34, where the increase in the pH of the sample solution, from 4.42 to 7.90, leads from no resolution to very well resolved peaks.

4. Effect of Buffer Concentration in the Sample Solution

An increase in the buffer concentration resulted in an increase in the retention time and in the substantial peak height increase for STB (Table XIX). While retention time increase was observed in the both, constant and increasing mass studies, the peak height enhancement was observed only in the increasing mass study. The possible explanation for this is that increase in the ionic buffer species can reduce the electrostatic repulsion between eluate molecules and increase the surface tension of the solvent. The solutions with higher solute concentations would experience greater effect and would therefore exibit reduced band broadening. The concentration dependent influence on the peak height of STB may also be due to the interaction of the STB molecules with the buffer species in the sample solution. As buffer concentrations increased, the interaction of phosphate anion with the amide proton of STB becomes greater. With the formation of STB-buffer complex, one would expect that the molecule would interact less strongly with the nonpolar stationary phase. As a result the retention time would decrease. The opposite was in fact true, as can be seen from the increase in retention

times observed when the buffer concentration increased. The most logical explanation for this is that the molecular conformation has been altered in such a way that butyl branch becomes a dominant factor in the retention mechanism. The nonpolar group has a stronger hydrophobic interaction with the stationary phase which results in a retention time increase. Another explanation could be that buffer species become adsorbed by the stationary phase and in this way alter retention mechanisms. This could also explain the behaviour of MBC. In this case a reduction in the retention time was observed as buffer concentrations increased, but peak height and peak width were not influenced. As the buffer concentration increased, more polar silanol groups became blocked by the adsorbed buffer species limiting silanol interaction with the polar MBC groups. Berthod et al. (91) reported behaviour similar to the results in this study for mobile phase effects in the study on surfactant concentrations. They found that the retention of neutral substances (caffeine, toluene) decreased when the amount of adsorbed surfactant was increased. When an anionic surfactant was adsorbed, the retention of negatively charged solutes, such as benzoate, fell sharply, and the retention of cationic substances, such as benzyltrimethyl ammonium bromide, increased. The cationic surfactant had opposite effect.

By maintaining the same buffer concentration, but changing the acetonitrile concentration from 5 to 35% different results were obtained for STB. At higer acetonitrile

concentrations retention times seemed almost constant while at lower acetonitrile concentrations an increase in the retenion was observed. The peak heights increased slightly more at a lower acetonitrile concentraion (26.5% for 10 µL injection at 5%), than at a higher concentration (19.7% for 10 µL injection at 35%) (Tables XVII, XVIII, XIX).

Another interesting observation was that as the buffer concentration in the sample solvent increased, the peak area of STB also increased. This was the only occasion that the peak area showed some response to the change in factors except in the case of peak splitting. The reason for this could be the presence of STB-buffer complex which may slightly enhance the absorbance readings at the wavelength used for this study. This conclusion was supported by a special series of experiments conducted to measure the UV spectra of STB sample solutions which were prepared with different solvent compositions. The results of the study indicated that absorbance increased as the buffer concentrations increased (Table XXI). The importance of keeping ionic strength of the mobile phase constant has been shown by others (30,90).The above results emphasize the importance of keeping a constant ionic strength in the sample solutions also.

C. Injection Volume

The reason for studying injection volumes was the fact

that column suppliers with microparticulate packings of 5 µm or less recommend using injection volumes of less than 10 µL for optimum chromatographic results (92). In practice, however, this advice is very often not observed and volumes of 50 and 100 µL are still routinely used. Several reasons exist for using larger injection volumes. For very dilute samples, such as in trace analysis, there is a limit in detector sensitivity for many existing older model systems and it is necessary to preconcentrate samples before chromatographic run. The possibility of using larger injection volumes would reduce the need for preconcentration. Limitations for UV detectors are also due to the detector cell size. For the Perkin-Elmer instrument used in the first part of this study, the detector cell size was 8 uL and if sample volume is much smaller, band broadening due to the large cell size will eliminate any chromatographic efficiency obtained using smaller injection volumes. In some cases of trace and residue analyses, samples must be completely dissolved and total quantity injected. Dissolution is very dificult in volumes smaller than 50 to 100 µL. It was also of interest to see how sample compositions influence peak profiles at different injection volumes. This aspect was investigated in this study by preparing two series of samples. The influence of injection volume at constant injection mass was studied by injecting 10, 50, 100, and 200 µL of successively more dilute sample solutions of 5, 1, 0.5, 0.25 µg/mL, respectively. In this case the same amount of analyte was introduced onto the column at

all four injection volumes. The influence of injection volume at increasing mass was studied by injecting 10, 50, 100, and 200 µL of sample solution at a constant concentration of 5 µg/mL. In this case an increasing amount of analyte was being injected onto the column with increasing injection volume.

The profile of the injected band is characterized by the injection volume (30). It was shown (30) that the band variance (σ^2) obeys following relationship:

$$\sigma^2 = V^2/\lambda^2 \qquad \text{eq. [8]}$$

where V is the sample volume and λ is a numerical factor which depends on the mode of injection, with the usual value of 2. It was also shown that the maximum sample volume V_m is proportional to the maximum detector cell volume V_{dm} and to the $(t_r)^{1/2}$. The effects of the injection volume on the chromatographic efficiency (N), where $N = (t_{ri}/\sigma)^2$, was reported in (93). This group found that at constant mass an increase in the injection volume from 10 to 500 µL resulted in a substential decrease in the chromatographic efficiency. In our study we found that the composition of the sample solvent had a greater influence on the chromatographic efficiency than did injection volume as can be observed from Figures 66 and 67. It could also be seen from these Figures that the efficiency does not necessarily decrease with the injection volume increase from 10 to 200 µL. In Figure 66, we can observe that at 5% acetonitrile in the sample solvent (at increasing mass study) the efficiency of both analytes increased with the increase in the injection volume. At 50%

Table XXIII*

Chromatographic Efficiency(N)** at Increasing Injection Volume
for STB and MBC at 5 and 50%(v/v) Acetonitrile in the Sample Solvent

Injection Volume µL	EFFICIENCY (N) STB 5% CH_3CN	STB 50% CH_3CN	MBC 5% CH_3CN	MBC 50% CH_3CN
10	2250.39	1178.75	2398.11	1766.05
50	2724.14	433.23	2148.07	927.03
100	3112.04	334.04	2397.45	526.08
200	3116.63	320.32	2524.68	202.17

*for Increasing Mass Study
**$N=5.52[tr/w(h/2)]^2$

Table XXIV*

Chromatographic Efficiency(N)** at Increasing Injection Volume
for STB and MBC at 5 and 50%(v/v) Methanol in the Sample Solvent

Injection Volume µL	EFFICIENCY (N) STB 5% CH_3OH	STB 50% CH_3OH	MBC 5% CH_3OH	MBC 50% CH_3OH
10	1226.94	1150.30	1093.74	1030.70
50	1251.79	2042.56	1368.58	1146.02
100	1528.18	2254.55	1510.43	1098.87
200	1833.42	2001.26	1855.33	881.60

*for Constant Mass Study
**$N=5.52[tr/w(h/2)]^2$

Figure 66: Plot of Efficiency (N) vs. Injection Volume for STB and MBC at 5 and 50% (v/v) Acetonitrile

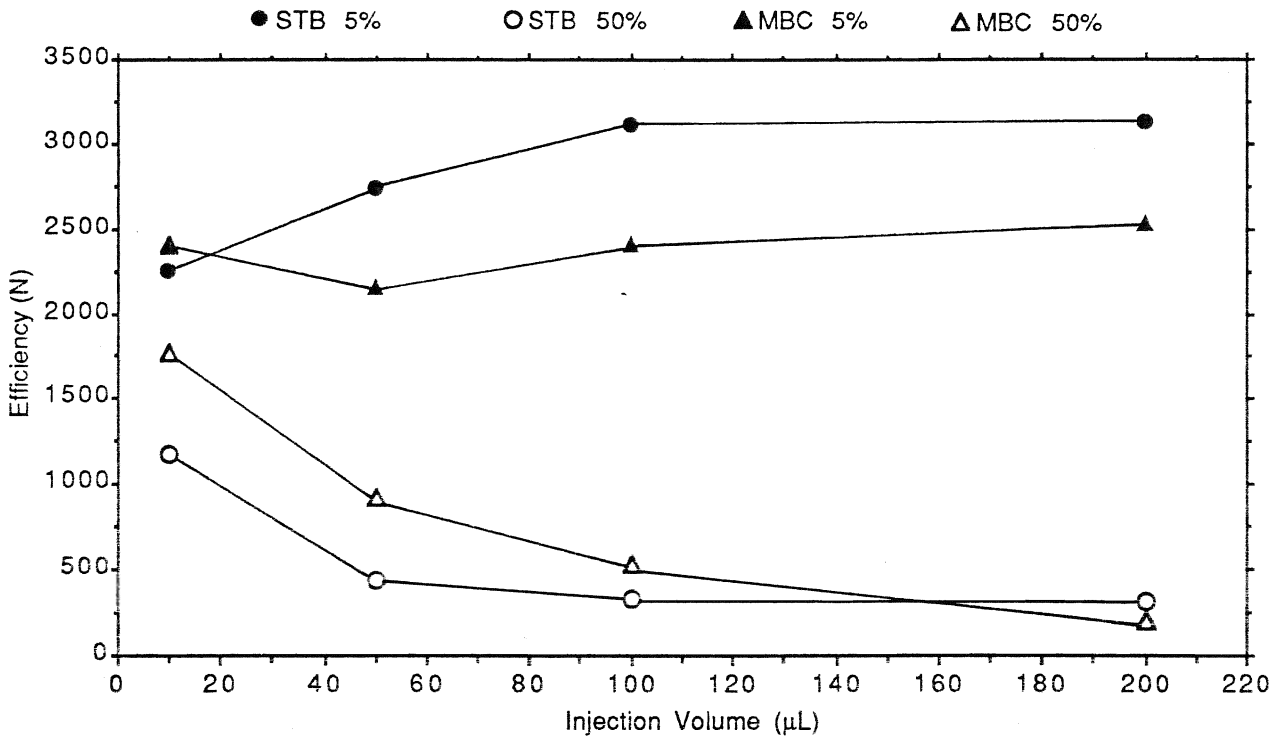
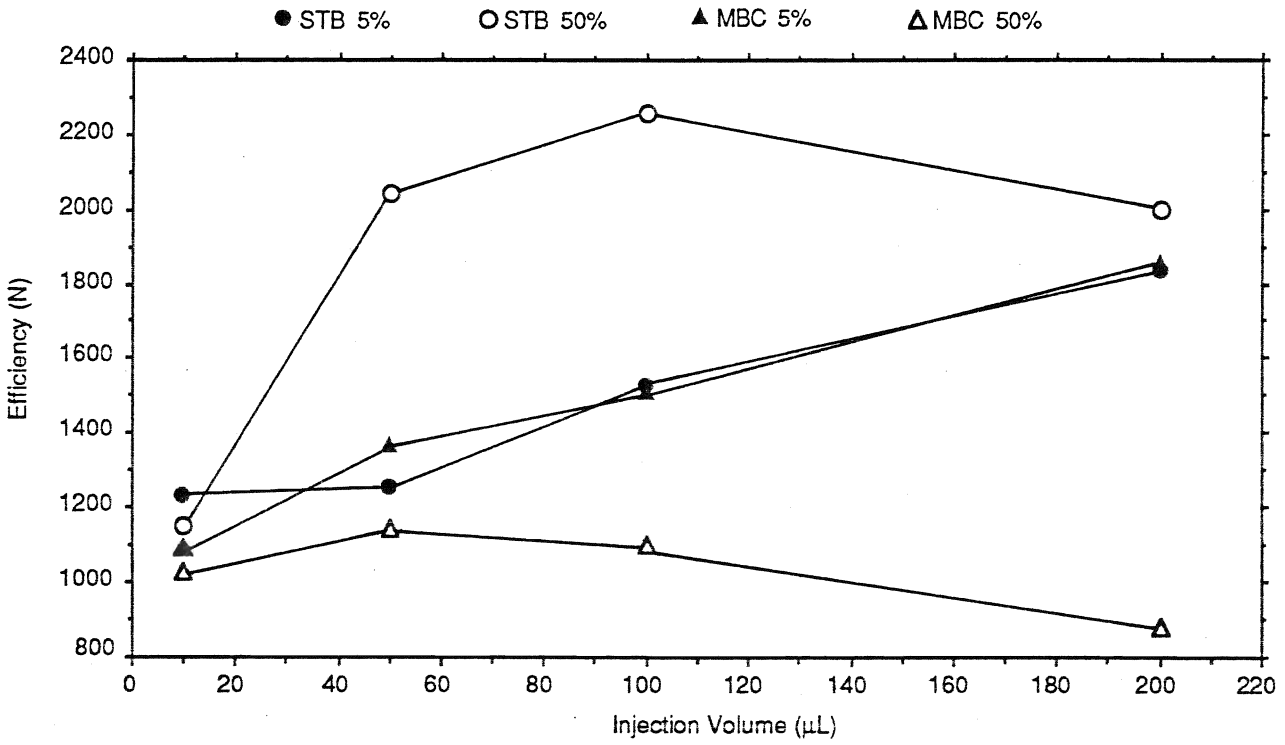


Figure 67: Plot of Efficiency (N) vs. Injection Volume for STB and MBC at 5 and 50% (v/v) Methanol



acetonitrile in the sample, completely opposite results were obtained. Here decreases in the efficiency, of similar magnitudes, were observed for both compounds. Somewhat different results were obtained for the methanol study (Figure 67). While at 5% methanol, similar efficiency increases were observed for both compounds, at 50% methanol, a pronounced increase was observed for STB while for MBC, efficiency gradually decreased at higher injection volumes.

In discussing chromatographic efficiency, one has to remember that it is a function of retention time (t_r) and the peak width ($w=4\sigma$). It should therefore be pointed out that as the injection volume increased the retention time of the analytes also increased. This increase contributes directly to the increase in the efficiency. Pronounced increases in the analyte retention times were observed at increasing injection volumes for lower percentage of the organic solvent than for the higher percentage (Tables IX-XIX). The larger increases in the efficiency at lower organic solvent content were also observed. At lower organic solvent contents the peak broadening, which is inversly proportional to the efficiency, was minimal or it actually decreased. At a higher organic solvent content, the differences in the retention times at various injection volumes became smaller and the peak broadening, which was qite substantial at the high organic solvent concentrations, became a dominant factor in controlling efficiency.

The effect of injection volume was more clearly observed

with acetonitrile than with methanol. Both retention time and peak height decreased when organic solvent concentrations increased and this trend was more pronounced with an increase in the injection volume (Figures IX-XI), but the magnitude of the effect was different for STB and MBC. Almost identical results were observed for the studies at constant and increasing mass, when the injection volume was low (at 10 and 50 µL). When the sample solvent strength was much lower than that of the mobile phase, the peak width was not significantly affected by an increase in the injection volume. However, when the sample solvent strength was higher than that of the mobile phase, the peak height became substantially smaller, this is particularly true when larger volumes of injections were made (Figure IX-XI). Peak splitting was often observed at 100 and 200 µL injections in the increasing mass study (Table Xa). The above observations are quite important since they suggest that by optimizing sample solvent composition a much larger injection volume can be used.

The effects of an increasing methanol concentration in the sample solvent on the peak profile were not as straight forward as those for acetonitrile. For MBC, decreasing trends in the retention time and peak height were observed as the injection volume increased, but this trend was not as pronounced as that for the acetonitrile. For STB, increase in the injection volume gave mixed results for methanol increase. In the constant mass study, in which successively less concentrated samples were injected, increase in the efficiency

in relation to the methanol percentage increase was very insignificant with 10 µL injections, but substantial increase was observed with 50 µL injections. This increase became less significant again with 100 and 200 µL injections. At a 200 µL injection volume and 50% methanol in the sample solvent a complete reversal in the peak height and efficiency was observed. The reason that the effects became smaller at higher injection volumes was most probably due to the presence of several effects on the retention mechanism of STB at the start of the analysis, which changed in the importance with the increasing injection volume. These effects were discussed in section IVB2.

For the increasing mass study, where successively larger quantities of analytes were injected at increasing volumes, different effects were observed. The increases in the retention time were larger than those for the constant mass study, at higher injection volumes. The peak height increases and the peak width decreases were observed only for 10 and 50 µL injections. At higher analyte concentrations, pronounced peak splitting and peak broadening were observed for 100 and 200 µL injections when methanol concentrations were higher than 5%. This suggests that at higher analyte concentrations, much smaller injection volumes can be tolerated for STB, if more than 5% of methanol is used in the injection solvent.

In the pH study, the sample solvent effects on the peak profile of STB became more pronounced with an increase in the injection volume, while for MBC, injection volume increases

had no effect on the peak profile.

In the buffer concentration study, analyte concentrations appeared to be an important factor. In the study where sucessively lower concentrations of samples were injected at higher volumes, injection volume effects were almost nonexistent. In the increasing mass study, increase in the injection volume had no pronounced effect on the peak heights and retention times for 10, 50 and 100 µL injections. At 200 µL injections, increases in peak heights and retention times for STB which were observed at lower injection volumes was replaced by peak height and retention time decreases (Tables XIXa and XIXb).

V. Conclusion

From the results of this study several important conclusions can be drawn about the effects of the sample solvent composition on the chromatographic peak profile.

1. The amount and the type of organic solvents, the pH, and the buffer strength in the sample solution can have a pronounced effect on the peak heights and the retention times of the compounds analysed.
2. Compounds that have low tendency to interact with the sample solvent, are not prone to ionization, or are capable of forming intramolecular hydrogen bonds show a predictable decrease in retention time and peak height and greater peak

broadening with an increase in the eluting strength of the sample solvent.

3. Compounds that have a tendency to ionize or to strongly interact with the sample solvent molecules may be influenced in various ways by the changes in the sample solvent composition and these type of compounds are influenced to a much greater extent by the pH and buffer strength variations in the sample solvent.

4. Effects of sample solvent on peak profiles can be different even for very similar group of compounds. For this reason, solvent compositions for both samples and standards should be kept constant; this is true even if the internal standard is used in the analysis.

5. Use of the mobile phase for sample preparation does not always result in the most efficient chromatograms and whenever possible samples for reversed phase HPLC analyses should be prepared with the least amount of organic solvent.

6. The effect of sample solvent compositions can be observed even with 10 µL injections. The effects, however, become more pronounced as injection volume increases. For this reason the smallest possible injection volume should be used if careful control of sample solvent composition is not possible.

7. By optimizing sample solvent compositions, much larger injection volumes may be used without significant loss of chromatographic efficiency.

8. The concentration of analytes becomes more important in the sample preparation if the analyte is capable of ionization or

has a strong interaction with the solvent.

VI. Recommendations

This study clearly demonstrated that the sample solvent composition can have pronounced effects on the chromatographic peak profiles. For this reason it is strongly recommended that all the samples and standards, regardless whether standards are external or internal, be prepared in a solution with the least percentage of organic solvent and analysed at a constant sample composition and a constant injection volume.

References

1. M. Tswett, Trav. Soc. Nat. Warsovie, 14 , (1906), translated and quoted in J. Chem. Ed., 44 , 235 (1967).

2. A.J.P. Martin and R.L.M. Synge, Biochem J., 35 , 194 (1941).

3. R. Wood, Biochem. Arch., 2 , 63 (1986).

4. A. J. Speek, C. R. Temalilwa and J. Schrijver, J. Food Chem., 19 , 65 (1986).

5. C. P. Tsai, A. Sahill, J. M. McGuire, B. L. Karger and P. Vouros, Anal. Chem., 58 , 2 (1986).

6. L. R. Snyder and J. J. Kirkland, Introduction to Modern Liquid Chromatography , Wiley-Interscience, New York, 1974, p.2.

7. M. A. Johnston, A. R. Lea and D. M. Hailey, J. Chromatogr., 318 , 362 (1985).

8. S. U. Sheikh and J. C. Touchstone, Anal. Chem., 59 , 1472 (1987).

9. H. Stray, A. Mikalsen, B. Soenstreud and M. Oehme, J. Chromatogr., 349 , 331 (1985).

10. R. J. Bushway, J. Chromatogr., 303 , 263 (1984).

12. J. J. Lesec, Liq. Chromatogr., 8 , 875 (1985).

12. G. J. DeJong, N. Summers, F. J. Spruit, C. Dewaele and M. Verzele, Anal. Chem., 59 , 1458 (1987).

13. J. J. Gogel and K. Biemann, Anal. Chem., 59 , 1266 (1987).

14. J. B. F. Loyd, Anal. Chem., 59 , 1401 (1987).

15. D. A. Catlow, J. Chromatogr., 323 , 164 (1985).

16. C. F. Simpson, Techniques in Liquid Chromatography , Wiley Heyden Publication, New York, 1982, p.270.

17. L. R. Snyder and J. J. Kirkland, Introduction to Modern Liquid Chromatography , Wiley-Interscience, New York, 1974, p.31.

18. W. A. Moats, J. Chromatogr., 366 , 69 (1986).

19. W. J. Adams, B. E. Bothwell, W. M. Bothwell, G. J. Vangiessen and D. G. Kaiser, Anal. Chem. 59 , 1504 (1987).

20. A. Marcomini and W. Giger, Anal. Chem., 59 , 1709 (1987).

21. N. Miller, H. E. Pretorius and D. Trinder, A. Assoc. Off. Anal. Chem., 68 , 136 (1985).

22. M. Friedman and and A. T. Noma, J. Agric. Food Chem., 34 , 497 (1986).

23. T. D. Spittler, R. A. Marafloti and L. M. Lahr, J. Chromatogr., 317 , 527 (1984).

24. G. Hoogewijs and D. L. Massart, J. Parm. Biomed. Anal. 2 , 449 (1984).

25. K. Takami, K. Kuwata, A. Sugimae and M. Nakamoto, Anal. Chem., 57 , 243 (1985).

26. T. T. Nguyen, P. Sporns and D. Hadziyev, J. Chromatogr., 363 , 361 (1986).

27. J. C. Moore, J. Polym. Sci., 21 , 119 (1964).

28. G.A. Howard and A. J. P. Martin, Biochem. J., 46 , 532 (1950).

29. H. N. M. Stewart and S. G. Perry, J. Chromatogr., 37 , 97 (1968).

30. C. Horvath, High-Performance Liquid Chromatography, Advences and Perspective , Academic Press, Vol. 2, New York, 1980, p.138.

31. R. P. W. Scott and C. F. Simpson, J. Chromatogr., 197 , 11 (1980).

32. P. Raumeliatis and K.K. Unger, J. Chromatogr., 149 , 211 (1978).

33. P.C. Sadek and P. W. Carr, J. Chromatogr. Sci., 21 , 314 (1983).

34. N. Cook and K.Olsen, J. Chromatogr. Sci., 18 , 512 (1983).

35. R. Bij, A. Nahum, W. Melander and C. Horvath, J. Chromatogr., 203 , 65 (1980).

36. D. MacBlane and J. R. Benson, Interaction Chemicals Inc., 1615 Plymouth Street, Mountain Veiw, USA.

37. J. Stahalberg and M. Almgren, Anal. Chem., 57 , 817 (1985).

38. C. H. Lachmuller, A. S. Calborn, R. L. Hunnecut and J. M. Harris, Anal. Chem., 55 , 1344 (1983).

39. J. F. Lawerence, Organic Trace Analyses by Liquid Chromatography , Academic Press, New York, (1981) p.34.

40. C. Horvath and W. Melander, J. Chromatogr. Sci., 15 , 393 (1977).

41. C. Horvath and W. Melander, Am. Lab., 10 , 11 (1978).

42. R. P. Scott and P. Kucera, J. Chromatogr., 142 , 213 (1977).

43. R. P. Scott and C. F. Simpson, Faraday Symposia of the Chem. Soc., 15 , 69 (1981).

44. R. P. Scott and C. F. Simpson, J. Chromatogr., 197 , 13 (1980).

45. N. Tanaka, Y. Takuda, K. Swagurki and M. Arabi, J. Chromatogr., 239 , 761 (1982).

46. H. McNair and J. Bowemaster, J. High Resol. Chrom. Comm., 10 , 27 (1987).

47. W. Melander, A. Nahum and C. Horvath, J. Chromatogr., 185 , 129 (1979).

48. J. B. Li, J. Analysis and Purification, 78, Feb. (1987).

49. L. R. Snyder, J. Chromatogr. Sci., 16 , 223 (1978).

50. P. J. Schoenmakers, H. A. H. Belliet, R. Tijsen and L. De Galon, J. Chromatogr., 149 , 519 (1978).

51. J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, J. Chromatogr., 199 , 57 (1980).

52. S. R. Abbot, J. R. Berg, P. Achener and R. L. Stevenson, J. Chromatogr., 126 , 421 (1976).

53. C. Horvath, W. Melander and I. Molnar, J. Chromatogr., 125 , 129 (1976).

54. B. L. Karger, J. R. Gant, A. Harthoph and P. H. Weiner, J. Chromatogr., 128 , 65 (1976).

55. N. Tanaka, H. Goodell and B.L. Karger, J. Chromatogr., 158 , 233 (1978).

56. A. Sokolwski and K. G. Wahlund, J. Chromatogr., 189 , 299 (1980).

57. A. Leitold and G. Vigh, J. Chromatogr., 257 , 384 (1983).

58. T. Ishimitsu and S. Hirose, Talanta., 32 , 865 (1985).

59. W. R. Melander, J. Stovaken and C. Horvath, J. Chromatogr., 185 , 111 (1979).

60. E. Pepp and G. Vigh, J. Chromatogr., 282 , 59 (1983).

61. J. F. Lawerence, Organic Trace Analysis by Liquid Chromatography , Academic Press, New York, 1981, p.34.

62. L. R. Snyder and J. J. Kirkland, Introduction to Modern Liquid Chromatography , Wiley-Interscience, New York, 1979, p. 290.

63. M. Tsimidou and R. Macrae, J. Chromatogr., 285 , 178 (1984).

64. D. A. Laude Jr. and C.L. Wilkins, Anal. Chem., 59 , 546 (1987).

65. M. Chiba and R. P. Singh, J. Agric. Food Chem., 34 , 108 (1986).

66. P. K. Tseng and L. B. Rogers, J. Chromatogr. Sci., 16 , 436 (1978).

67. K. J. Williams, A. Won Po Li and W. J. Irwin, J. Chromatogr., 194 , 217 (1980).

68. M. Tsimidou and R. Macrae, J. Chromatogr. Sci., 23 , 155 (1985).

69. T. L. Ng and S. Ng, J. Chromatogr., 329 , 13 (1985).

70. S. Perlman and J. J. Kirschbaum, J. Chromatogr., 357 , 39 (1986).

71. L. B. Nilsson, D. Westerland, Anal. Chem., 57 , 1835 (1985).

72. J. J. Kirschbaum, S. Perlman, J. Adamovic and J. Joseph, J. Chromatogr. Sci., 23 , 161 (1985).

73. S. Levin and E. Grushka, Anal. Chem., 58 , 1602 (1986).

74. S. Levin and E. Grushka, Anal. Chem., 59 , 1157 (1987).

75. D. Berek, T. Bleka and Z. Pevna, J. Chromatogr. Sci., 14 , 560-63 (1976).

76. K. Slais and M. Krejci, J. Chromatogr. Sci., 91 , 161-166 (1974).

77. A. Rouchouse, D. Pauloin and J. T. Burke, J. Chromatogr., 353 , 225 (1986).

78. M. Chiba and D. F. J. Veres, Assoc. of Anal. Chem., 63 , 1291 (1980).

79. R. P. Singh and M. Chiba, J. Agric. Food Chem., 33 , 63 (1985).

80. P. Carbas, M. Meloni, M. Perra and F. M. Pirisi, J. Chromatogr., 180 184 (1979).

81. R. G. Bates, Determination of pH-Theory and Practice , Wiley-Interscience, New York, 1973, p. 243.

82. J. C. Berridge, J. Chromatogr., 369 . 265 (1986).

83. H. H. Jaffe and M. Orchin, Theory and Applications of Ultraviolet Spectroscopy , Wiley-Interscience, New York, 1966, p.18.

84. S. Edawrds and J. F. Hinton, Solvent Effects on Chemical Phenomena , Academic Press, New York, 1973, p.337.

85. C. N. Rao, Ultra-Violet and Visible Spectroscopy, Chemical Applications , Butterworth, London, 1961, p 71.

86. J. J. Kirschbaum and S. Perlman, J. Chromatogr., 369 , 269 (1986).

87. T. Braumann, J. Chromatogr., 337 , 191 (1986).

88. E. V. Titova, M. A. Chlenov and L. I. Kudjashov, J. Chromatogr., 364 , 209 (1986).

89. H. Colin, J. C. Diez-Masa, G. Guichon, T. Czajkowska and I. Medziak, J. Chromatogr., 167 , 41 (1978).

90. J. P. Foley and W. E. May, Anal. Chem., 59 , 102 (1987).

91. A. Berthod, I. Girard and C. Gonnet, Anal. Chem., 58 , 1359 (1986).

92. N. H. C. Cooke, B. C. Archer, K. Olsen and A. Berick, Anal. Chem., 54 , 2277 (1982).

93. Waters-Canada, HPLC School, Gs-16&17, 1983.

Appendix I

Effects of Sample Solvent Composition on the Peak Profiles of STB and MBC obtained on PE-HPLC

Composition of Sample Solutions for Acetonitrile Concencentration Study on PE-HPLC

% CH_3CN	% CH_3OH	% 0.07M Buffer at pH	[STB] µg/mL	[MBC] µg/mL	% H_2O
5.00	5.00	10.00	5.00	5.00	80.00
25.00	5.00	10.00	5.00	5.00	60.00
35.00	5.00	10.00	5.00	5.00	50.00
50.00	5.00	10.00	5.00	5.00	35.00

Effect of Increasing CH_3CN Concentration in the Sample Solvent from PE-HPLC Study*

CH_3CN %	Retention Time (min) STB	Retention Time (min) MBC	Peak Height (cm) STB	Peak Height (cm) MBC
5.00	3.40+/- 0	4.00+/- 0	9.30+/-.05	12.51+/-.06
25.00	3.30+/-.02	3.88+/-.02	8.74+/-.09	11.94+/-.10
35.00	3.20+/-.02	3.88+/-.04	8.56+/-.02	11.56+/-.02
50.00	3.20+/-.04	3.82+/-.02	6.39+/-.01	9.77+/-.03

* at 50 µL injection volume

Composition of Sample Solutions for Methanol Concencentration Study on PE-HPLC

% CH_3OH	% CH_3CN	% 0.07M Buffer at pH 7	[STB] µg/mL	[MBC] µg/mL	% H_2O
5.0	5.0	10.0	5.0	5.0	80.0
25.0	5.0	10.0	5.0	5.0	60.0
35.0	5.0	10.0	5.0	5.0	50.0
50.0	5.0	10.0	5.0	5.0	35.0

Effect of Increasing CH_3OH Concentration in the Sample Solvent on STB and MBC from PE-HPLC Study*

[CH_3OH] %	Retention Time (min) STB	Retention Time (min) MBC	Peak Height (cm) STB	Peak Height (cm) MBC
5	3.20+/- 0	4.00+/- 0	12.82+/-.13	17.72+/-.24
25	3.20+/- 0	4.00+/- 0	12.88+/-.03	17.50+/-. 0
35	3.18+/-.02	4.00+/- 0	13.27+/-.06	17.50+/-.25
50	3.18+/-.02	4.00+/- 0	13.57+/-.42	17.48+/-.59

* at 10 µL injection volume

Composition of Sample Solutions for pH Study on PE-HPLC

% CH_3CN	% CH_3OH	0.07M Buffer	[STB] µg/mL	[MBC] µg/mL	H_2O %
5.00	5.00	10.00 at pH 5.00	5.00	5.00	80.00
5.00	5.00	10.00 at pH 6.00	5.00	5.00	80.00
5.00	5.00	10.00 at pH 7.00	5.00	5.00	80.00
5.00	5.00	10.00 at pH 8.00	5.00	5.00	80.00
5.00	5.00	10.00 at pH 9.00	5.00	5.00	80.00

Effect of pH Increase in the Sample Solvent on the Peak Profiles of STB and MBC in PE-HPLC Study*

pH	Retention Time (min)		Peak Height (cm)	
	STB	MBC	STB	MBC
5.00	3.68+/-.02	4.14+/-.02	8.07+/-.04	12.98+/-.03
6.00	3.66+/-.06	4.14+/- 0	8.33+/-.03	12.87+/-.03
7.00	3.56+/- 0	4.14+/- 0	9.30+/-.05	12.51+/-.06
8.00	3.46+/-.04	4.14+/-.06	9.78+/-.08	12.82+/-.08
9.00	3.48+/- 0	4.18+/-.02	8.71+/-.08	12.67+/-.06

* at 50 µL injection volume

Composition of Sample Solutions for Buffer Concentration Study on PE-HPLC

CH_3CN %	CH_3OH %	Buffer at pH 7.0	[STB] µg/mL	[MBC] µg/mL	H_2O %
5.00	5.00	10.00 at 1.200M	5.00	5.00	80.00
5.00	5.00	10.00 at 0.700M	5.00	5.00	80.00
5.00	5.00	10.00 at 0.070M	5.00	5.00	80.00
5.00	5.00	10.00 at 0.007M	5.00	5.00	80.00

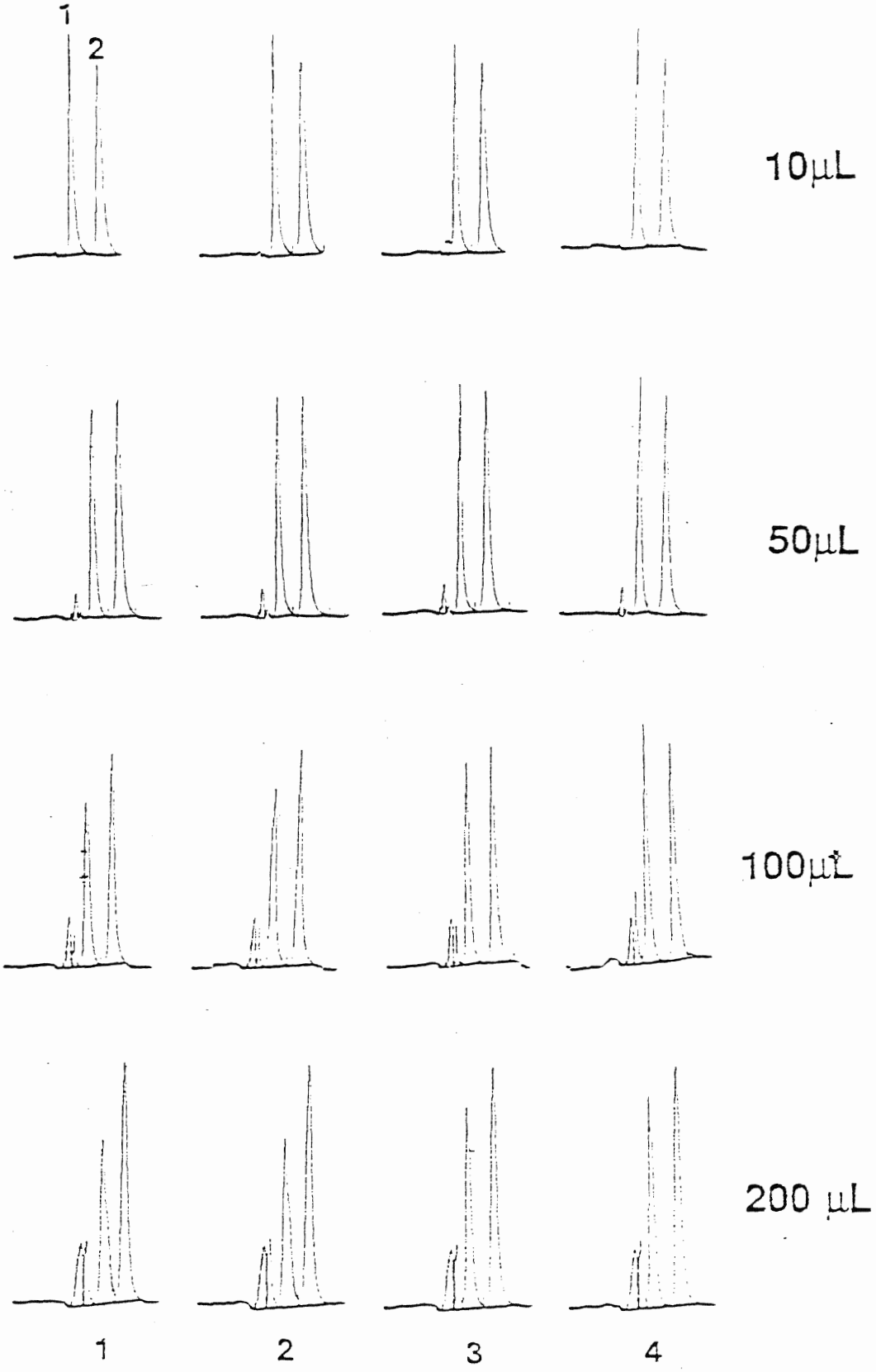
Effect of Buffer Concentration in the Sample Solvent on the Peak Profiles of STB and MBC from PE-HPLC Study*

[Buffer] M	Retention Time (min) STB	Retention Time (min) MBC	Peak Height (cm) STB	Peak Height (cm) MBC
0.0007	3.34+/-.02	4.06+/-.04	12.83+/-.2	17.55+/-.4
0.007	3.30+/- 0	4.08+/- 0	12.88+/-.07	17.72+/-.07
0.07	3.34+/-.04	4.10+/- 0	13.56+/-.5	17.62+/-.8
0.12	3.28+/-.02	4.04+/- 0	14.00+/-.5	17.36+/-.5

* at 10 µL injection volume

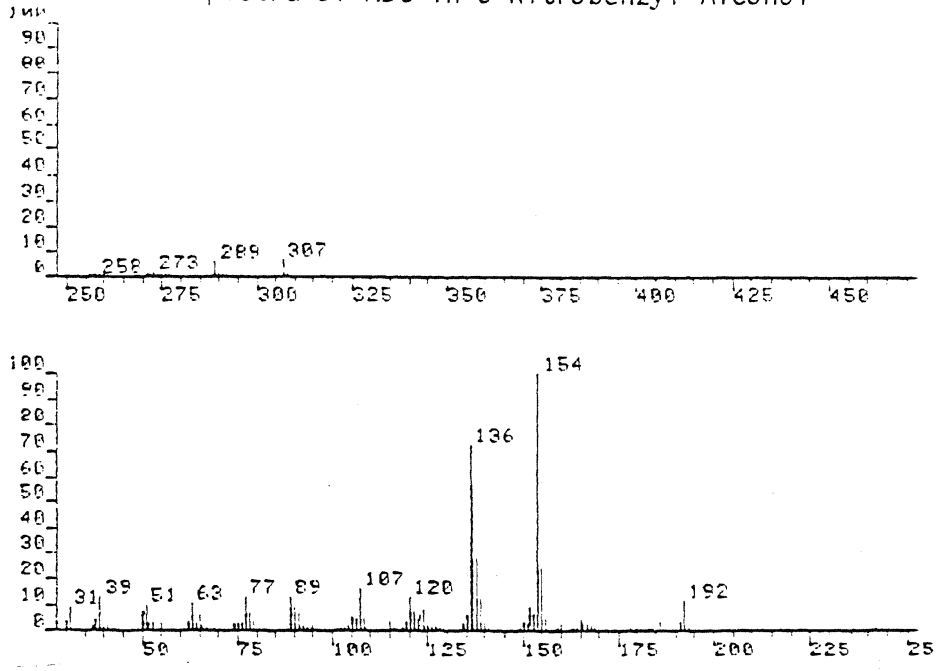
Appendix II

Chromatograms of STB (1) and MBC (2) for Constant Mass Study Showing the Effects of the Sample Solvent pH at Increasing Injection Volume.



Appendix III

FAB Mass Spectra of MBC in 3-Nitrobenzyl Alcohol



FAB Mass Spectra of STB in 3-Nitrobenzyl Alcohol